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GTPBP2 is a positive regulator of TGF-β signaling, and is required for embryonic patterning in Xenopus.

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Abstract of the Dissertation

GTPBP2 is a positive regulator of TGF-β signaling, and is required for embryonic patterning in Xenopus.

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The Transforming Growth Factor β (TGF-β) superfamily of signaling proteins regulate a diverse set of biological processes, including cell proliferation, adhesion, migration, apoptosis, differentiation and embryonic pattern formation. Because of its key role in these processes, a plethora of regulators are evolved to modulate TGF-β signaling. The TGF-β superfamily is comprised of about 30 ligands, which are commonly grouped into two broad sub-families based on their downstream signaling effectors: the TGF-β/Nodal/Activin and BMP/GDF families. To identify novel molecules that regulate the intracellular BMP/Smad1 signaling pathway, we have undertaken a yeast two-hybrid screen using Smad1, and retrieved GTPBP2 as a binding partner for Smad1. GTPBP2 and its close homolog GTPBP1 are large GTPases of unknown function. In this study, I have shown that GTPBP2 interacts with a subset of Smad proteins, and consistent with these interactions GTPBP2 induces mesoderm in explants and enhances canonical TGF-β signaling pathways in Xenopus and HepG2 cells. GTPBP2 mRNA is maternal and expressed in a dynamic pattern in developing Xenopus embryos. By knocking down GTPBP2 levels, I showed that GTPBP2 is required for mesendodermal patterning, and BMP signaling. GTPBP2 is a nuclear protein and colocalizes to nuclear foci with Smad1. In conclusion, these results show that GTPBP2 is a positive regulator of TGF-β signaling.

To Ayhan, Burcu Anil and Sevim Kirmizitas

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Chapter 1. Background and Significance

1.1 TGFβ signaling pathways

The Transforming Growth Factor β (TGF-β) superfamily of signaling proteins regulate a diverse set of biological processes, including cell proliferation, adhesion, migration, apoptosis, differentiation and embryonic pattern formation (Shi and Massague, 2003; ten Dijke et al., 2002; Hill, 2001; Schier and Talbot, 2005). The TGF-β super family is comprised of about 30 ligands, which are commonly grouped into two broad sub-families based on their downstream signaling effectors: the TGFβ/Nodal/Activin and BMP/GDF families (Ten Dijke and Hill, 2004). TGF-β ligands are synthesized as dimeric large precursors and proteolytically cleaved into a C-terminal mature peptide and a propeptide in the ER. (Gray and Maon, 1990; Annes et al., 2003) Mature ligand is a homodimer (e.g. TGFβ) or a heterodimer (e.g. BMP4/7) of two polypeptides joined by a disulfide bond (Yeo and Whitman, 2001). The ligands signal through cell surface complexes of type I and type II receptors. These two types are structurally similar transmembrane serine/threonine kinases, but type I receptors have an additional conserved juxtamembrane region called Glycine/Serine rich "GS" domain. Ligand binding allows formation of a stable a heterotetrameric receptor complex composed of two type II and two type I serine receptors. In this complex the type II receptors phosphorylate the type I receptors at GS domain, which activates the type I receptor resulting in autophosphorylation of type I receptor (Yamashita et al., 1994; Luo and

Lodish, 1996; Shi and Massague, 2003). The activated type I receptor relays the signal inside the cell through phosphorylation of R-Smads at their C-terminal SXS motifs.

The Smads are the principal intracellular effectors of TGF-β signaling. Smads are categorized in three subgroups; receptor regulated or R-Smads (Smad 1, 2, 3, 5, 8), common Smad or co-Smad (Smad4), and inhibitory Smads (Smad6 and 7). TGFβ/Nodal/Activin type ligand activated type I receptors; ALK4, ALK5, and ALK7 phorphorylate Smad2 and Smd3, whereas BMP/GDF type ligand activated ALK1, ALK2, ALK3, and ALK6 receptors phosphorylate Smad1, Smad5, and Smad8 (Attisano and Wrana, 2002). Smad proteins get recruited to activated receptor complexes by adaptor proteins such as SARA and endofin (Tsukazaki et al., 1998; Shi et al, 2007). R-Smads and Smad4 contain two conserved motifs; the MH1 domain at the N-terminus and the MH2 domain at the C-terminus linked by a less conserved linker domain. In the basal state, the MH1 and MH2 domains interact with each other and inhibit each others function; the MH2 domain represses MH1 mediated DNA binding, whereas MH1 domain inhibits MH2 mediated transactivation. C-terminal phosphorylation of R-Smads leads to a conformational change which trigger their dissociation from the receptor, and the formation of a trimeric complex consisting of two R-Smads and one Smad4 that translocates into the nucleus, where Smads act as transcription factors (Shi and Massague, 2003). Smads bind to DNA either with low affinity or not at all. Therefore, they require other sequence specific transcription factors to bind efficiently to the promoters of target genes (Feng and Derynck, 2005) (Figure 1.1).

Although the core pathway is linear and straightforward, because of its importance in embryonic development and tissue homeostasis, a variety of mechanisms

have evolved to regulate the boundary, intensity, duration and activity of TGFβ pathway. These mechanisms include use of competitive inhibitors in the extracellular matrix that are limiting ligand accessibility to the receptor, truncated receptor homologs at the cell membrane, inhibitory Smads, sequestering and, or degradation (Itoh and Dijke 2007). And the post-translational modifications on Smad proteins, which include ubiquitination, sumoylation, acetylation, linker region phosphorylation as well as carboxy terminal phosphorylation, have been shown to be critical for stability and transcriptional activity of Smads (Massague et al., 2005).

1.2 Regulation of TGFβ Signaling

1.2.1 Mechanisms that modulate signaling at ligand-receptor level

The regulation of some TGF-β ligands starts even before they are secreted as active dimers. For example, TGF-β1 ligand is secreted as an inactive complex, known as LTBP, in which two propeptides remain associated with ligands. It requires a further activation step to release the active ligand, which involves the metalloprotease, BMP-1 (Tolloid) (Ge and Greenspan, 2006). BMP1/TLD-like proteases also liberate BMP2 and 4 from latent complexes with the extracellular antagonist chordin. This results in the formation of the BMP signaling gradient that forms the dorsal-ventral axis in embryogenesis (Piccolo et al., 1997; Wardle et al., 1999).

After ligands are processed and secreted, they interact with a plethora of inhibitory proteins in a spatially and temporally regulated fashion. These inhibitors act as ligand sequestering proteins that prevent active ligands from binding to the receptors. Most of these ligands are BMP antagonists, including chordin, noggin, cerberus, gremlin,

twisted gastrulation, and sclerostin. Cerberus additionally inhibits nodal/activin type TGF-β ligands and Wnt proteins. Lefty is an inhibitor of Nodal ligands and Follistatin which is originally isolated as an activin inhibitor also binds BMPs. (Meno et al., 1998; Dal-Pra et al., 2006; Harrington et al., 2006; Schmierer and Hill, 2007). Dynamic expression patterns, diffusion capacity, and stability of these antagonists allow modulation of the strength of TGF-β signaling in a temporal and spatial manner. A subset of ligands require the presence of co-receptors (type III receptors), which enhance the affinity of ligand-receptor (type I / II receptor) interaction or present the ligand to the receptor. Examples of type-III receptors include betaglycan, biglycan, endoglin, and EGF-CFC/cripto (Lopez-Casillas et al, 1991; Moreno et al., 2005; Scherner et al., 2007). EGF-CFC is a good example of how type III receptors differentially regulate signaling. TGF-β ligands activin and nodal signal by binding to the same receptors. However, nodal strictly depends on the presence of EGF-CFC co-receptors to signal (Gritsman et al., 1999).

Signaling downstream activated receptors is inhibited by pseudoreceptor BAMBI. BAMBI has a high similarity to TGF-β receptors but lack the intracellular Ser/Thr kinase domain, and acts as dominant negative repressor (Onichtchouk, 1999). Another example of modulation at receptor level is receptor tyrosine kinase; Ror2. Ror2 binding to BMP type I receptor inhibits phosphorylation of Smad1/5 (Sammar et al., 2004).

Inhibitory Smads, Smad6 and Smad7 are induced in response to TGF-β ligands and act as dominant negative repressors in many contexts. I-Smads bind to type-I receptors and interfere with their binding to R-Smads, reducing signaling downstream of activated receptors (Hayashi et al., 1997). Smad7 interacts constitutively with ubiquitin

ligases Smurf1 and Smurf2, and targets them to the activated receptor, which leads to degradation of active receptors via lipid raft-caveolar internalization pathway (Kavsak et al., 2000; Ebisawa et al., 2001; Di Guglielmo et al., 2003). Smad7 is also involved in targeting of GADD34, a regulatory subunit of protein phosphatase-1 (PP-1) to active receptors, which leads to their inactivation by dephosphorylation (Shi et al., 2004).
In addition to the competitive binding to type I receptors, Smad6 also inhibits complex formation between BMP activated Smad1 and Smad4, by binding to Smad4 (Hata et al., 1998).

1.2.2 Mechanisms that modulate signaling at Smad level

Efficient R-Smad recruitment and activation in response to TGF-β ligands requires accessory proteins; SARA and endofin (Tsukazaki et al., 1998; Shi et al., 2007). SARA, and endofin are FYVE domain containing proteins that interact with Smad2/3 and Smad1/4/5 respectively. SARA is also shown to interact with type I receptor. SARA is localized at the plasma membrane and concentrated in early endosomes through interaction of FYVE domain with membrane lipids. Complex formation of the receptors with SARA and Smad2/3 in early endosomes is essential to efficiently initiate TGF-β signaling (Hayes et al., 2002; Di Gueglimo et al., 2003). Other adaptor proteins; Hsg, Disabled-2, PML are involved in facilitating receptor-Smad interaction (Miura et al., 2000; Hocevar et al., 2001; Lin et al., 2004). Receptor mediated phosphorylation causes a decrease in the affinity of Smad2 for SARA that is sufficient for Smad2/3 dissociation and movement into the nucleus (Wu et al., 2000).

R-Smads and Smad4 are present mostly in the cytoplasm or evenly throughout the cell but become concentrated in the nucleus upon TGF-β stimulation. The amount of Smads accumulated in the nucleus and the duration of Smads being associated with the promoters are critical determinants of transcriptional outcomes (Wilson et al., 1997). Therefore, nucleocytoplasmic translocation of Smads is an important step of TGF-β regulation. The nuclear translocation of Smads is simple, and done by direct interaction with nuclear pore proteins, nucleoporins through exposed MH2 domain in phosphorylated R-Smads (Xu et al., 2002). Transcriptional repressor SnoN is localized to cytoplasm and antagonizes TGF-beta signaling by sequestering Smad2/3/4 in the cytoplasm (Krakowski et al., 2005). xMAN1, an inner nuclear membrane protein, sequesters R-Smads at the nuclear envelope, causes disassembly of R-Smad-Smad4 complexes, dephosphorylation of Smads, and increased nuclear export, thus prevents transcription from their target genes (Raju et al., 2003; Bengtsson, 2007).

1.2.3 Modulation of Smad activity by covalent modifications

Phosphorylation and Dephosphorylation

Phosphorylation of the C-terminal serine residues of R-Smads by activated receptor is not the only phosphorylation event controlling Smad activity. The variable linker region in R-Smads contains multiple serine and threonine consensus sites for MAP kinases and praline directed kinases, allowing for regulation of R-Smads by multiple signaling inputs.

The linker region of Smad1 consists of four MAPK phosphorylation sites, whereas Smad2/3 consist of four SP/TP sites for proline-directed kinases (Sapkota et al.,

2006). ERK MAPK mediates the phosphorylation of these sites in vivo (Kretzschmar et al., 1997 & 1999). CDK2 and -4 have also been reported to mediate the phosphorylation of the linker residues in Smad2/3 (Matsuura et al., 2004). Linker phosphorylation of Smad1 by MAPK and consequent GSK3 mediated secondary phosphorylation of the adjacent residues N-terminal to MAPK sites in the linker region promotes interaction with ubiquitin ligase Smurf1 which induces Smad1 polyubiquitination and proteosome-dependent degradation (Zhu et al., 1999; Sapkota et al., 2007; Fuentealba et al., 2007). GSK3-β mediated phosphorylation is also involved in Smad3 ubiquitination and degradation (Guo et al., 2008). Linker phosphorylation of Smad2 by MAPK results in cytosolic retention of Smad2/3 and inhibition of TGF-β signaling (Grimm and Gurdon, 2002). p38 MAPK and JNK also phosphorylate the linker region of Smad2/3 and are necessary for the full transcriptional activation potential of Smad2/Smad3 by TGF-β (Mori et al, 2004; Kamaraju and Roberts, 2005**).**

In addition to linker phosphorylation mediated degradation of Smads, TGF-β signaling can also be terminated via phosphatases that catalyze C-terminal serine dephosphorylation (Inman et al., 2002). The first examples of such activity, PDP1 and 2 dephosphorylate Smad1, but not Smad2 and 3 (Chen et al., 2006), whereas PPM1A a general phosphatase with multiple targets, dephosphorylate Smad1, 2 and 3 (Lin et al., 2006; Duan et al., 2006). Small C-terminal domain phosphatases (SCP1, 2, and3) are also shown to dephosphorylate Smad1 C-terminal tail (Knockaert, et al. 2006). However, further studies pointed out a primary role for SCPs in dephosphorylation of linker regions of Smad 1, 2 and 3, leading to enhancement of TGF-β signaling, while at the same time resetting Smad1 to its basal unphosphorylated state (Sapkota et al, 2006) (Figure 1.2).

Ubiquitination and Sumoylation

Ubiquitin-dependent protein degradation is an important mechanism to regulate protein function. R-Smads phosphorylated by TGF-β receptors undergo ubiquitination and subsequent degradation (Lo and Massague, 1999). The steady level of R-Smads is also subject to stability control by ubiquitination, which may limit the extent of TGF-β response in cells with high levels of ubiquitin ligases (Zhu et al., 1999). Smurf1 is a HECT domain E3 ubiquitin ligase that contains an N-terminal C2 domain, two WW domains and a carboxy-terminal HECT domain that catalyses the transfer of the ubiquitin to its target substrates. The WW domains of Smurf1 bind to PY motif of the BMP-regulated Smad1/5, thereby allowing Smurf1 to target Smad1 for ubiquitination and proteasomal degradation (Zhu et al., 1999). In addition to Smurf1, other E3 ubiquitin ligases; Smurf2, Nedd4-2, SCF/Roc1, and WWP1 also mediate polyubiquitination and degradation of R-Smads through PPXY motifs (Lin et al., 2000; Kuratomi et al., 2005; Fukuchi et al, 2001; Komuro et al., 2004). Smad4 lacks a PPXY motif to recruit Smurfs. Smad4 is polyubiquitinated by SCF (beta-TrCP1), and ectodermin (Wan et al., 2004; Dupont et al., 2005). The inhibitory Smad, Smad7 can be targeted for ubiquitination by the RING-domain E3 ligase, Arkadia (Koinuma et al., 2003). Interestingly, Smad7 can also bind to Smurfs, and acts as a cofactor promoting receptor degradation without being ubiquitinated by itself (Kavsak et al., 2000; Gronroos et al., 2002). Smad7 is protected from ubiquitination via acetylation of the Smurf target lysine residues by p300 acetyltransferase (Simonsson et al, 2005). SIRT1, a histone deacetylase, reverses p300 mediated acetylation of Smad7; enhancing Smurf mediated ubiquitin proteasome

degradation (Kume et al., 2007). Interestingly, ubiquitination is also shown to affect TGF-β signaling in a positive way. Itch, an E3 ubiquitin ligase, promotes Smad2 ubiquitination without an apparent effect on Smad2 stability and degradation. In Itch null cell lines, sensitivity to TGF-β stimulation is decreased, with reduced Smad2 phosphorylation and nuclear localization. It was proposed that ubiquitinated Smad2 interacted better with TGF-β receptor complex, resulting in enhanced TGF-β signaling (Bai et al., 2004) (Figure 1.2).

Sumoylation is a process similar to ubiquitination in which SUMO (small ubiquitin like modifier) is linked to the lysine residues of target proteins by E3 SUMO ligases. Rather than tagging proteins for proteosomal degaradation, sumoylation is shown to alter protein function by creating a new interaction domain (Song et al., 2004). PIAS E3 ligase mediated sumoylation of Smad4 is shown to affect its transcriptional activity in both positive and negative manner. PIAS stabilizes, and increases Smad4 levels possibly by competing with ubiquitination, thereby resulting in higher transcriptional activity (Lee et al., 2003; Lin et al., 2003). However, sumoylation also creates binding surface for Daxx, a transcriptional repressor, which represses Smad4 mediated transcription in cells expressing Daxx (Chang et al., 2005).

1.2.4 In the nucleus; modulation of Smad activity by co-factors

The intrinsic DNA affinity of Smads is relatively low and Smads can not directly recruit basal transcription machinery and thus can activate transcription on naked DNA templates, Smad transcription complexes can only activate transcription on chromatin templates (Ross et al., 2006). Therefore, Smads require other sequence specific

transcription factors to efficiently bind to promoters and recruit transcriptional complexes (Itoh and Ten Dijke, 2007). Examples include DNA binding transcription factors; FAST (interacts with Smad2), and OAZ (interacts with Smad1). The FAST and OAZ proteins cannot activate transcription on their own because they lack a transactivation domain. These proteins may therefore act as DNA-binding adaptors for the Smads (Chen et al., 1996; Hata et al., 2000). On the other hand, Smads also bind to transcription factors; TFE3, AP-1, ATF-2 (CREB), and AML1, that are themselves sufficient to drive transcription. In this case, Smad recruitment is believed to augment or modify the activity of an existing transcriptional complex (Massague and Wotton, 2000).

In addition to transcription factors, Smads have been shown to interact with a number of co-activators (histone acetylases; p300/CBP and GCN5) and co-repressors (Ski, SnoN, Sip1, TGIF) with chromatin-modifying activities and in addition can recruit components of chromatin-remodeling complex, SWI/SNF (Ross and Hill, 2008). For example, Ski and SnoN disrupt the interaction of the Smads with p300 and instead recruit a co-repressor complex consisting of N-CoR, mSin3A and HDAC1 (Akiyoshi et al., 1999; Luo et al., 1999). Thus, the outcome of TGF-β signaling depends on the availability of the co-activators and co-repressor present in the receiving cell.

1.3 GTPBP2, a large GTPase of unknown function

To identify novel molecules that regulate the intracellular BMP/Smad1 signaling pathway, a former graduate student, Haitao Zhu did a yeast two-hybrid screen using Smad1 as bait, and retrieved GTPBP2 as a binding partner for Smad1. GTPBP2 and its close homolog GTPBP1 are large GTPases of unknown function. It is well established

that GTPases are involved in various cellular functions, including control of cell proliferation and differentiation, intracellular transportation, regulation of cytoskeleton, and protein synthesis. GTPBP2 was first isolated through its interaction with cytoplasmic tail of BH-Protocadherin-C, and by its homology to GTPBP1 (Watanabe et al., 2000; Senju et al., 2000). These two studies are limited in scope to characterization of gene structure and expression analysis. In a third study, It is shown that translocations involving GTPBP2 is linked to glioblastoma formation (Mulholland et al., 2006).

GTPBP1 was isolated as an interferon gamma inducible gene in monocytic leukemia cell line, THP-1 (Senju and Nishimura, 1997). This study is also limited to expression pattern and basic characterization of gene structure. GTPBP1 was knocked-out in mice without an apparent phenotype (Senju et al., 2000).

GTPBP2 and GTPBP1 proteins have 49% identity and 66% similarity to each other. They share three conserved domains; a GTPase domain close to N-terminus followed by two domains; GTPBPII and GTPBP2III that have homology to translation elongation factor, eEF1A and its paralogs.

Canonical function of eEF1A is to facilitate peptide chain elongation during mRNA translation. However, eEF1A also has a wide array of other functions, including activation of signaling enzymes PLCγ1 and SK1 (Chang et al., 2002; Leclercq et al., 2008), regulation of actin cytoskeleton (Gross and Kinzy, 2005), and induction of apoptosis by acting as a transcriptional repressor (Rho et al., 2006). Paralogs of eEF1A; eRF3 (GSPT1-2) and HBS1 have functionally diverged and do not bind aa-tRNA (Inagaki et al., 2003). In addition to its role in translation termination, eRF3 is involved in mRNA degradation via its role in mRNA deadenylation (Funakoshi et al., 2007). GSPT1

induces apoptosis by activating ASK1, a member of MAPKKK family (Lee et al., 2008). HBS1 together with Dom34, an endonuclease, is involved in a quality control system (No-Go decay) that recognizes and degrades non-functional mRNAs (Doma and Parker, 2006).

1.4 Embryonic Development of Xenopus Laevis

Xenopus Laevis (South African Clawed Frog) has been used as a key model organism to understand the basics of embryonic development since 1950s. Originally, its ability to spawn when injected with pregnancy urine made Xenopus an ideal tool for human pregnancy tests (Polack, 1949). The ability to obtain eggs year round led investigators to consider its use in experimental embryology. Classical embryology experiments by Pieter D. Nieuwkoop and techniques developed by John B. Gurdon proved the versatility of Xenopus as a major model organism to understand embryonic pattern formation (Sudarwati, and Nieuwkoop, 1971; Gurdon et al., 1971). Using Xenopus as a model organism has many advantages. Among these, one can list, easiness of obtaining large number of eggs year round, and in vitro fertilization which results in abundance of stage matched embryos that can be analyzed live under microscope, large embryo size and robustness of the embryos which enables researchers to do explant and transplant assays easily, possibility of manipulating culture temperatures to slow down development for experimental procedures. However, due to its long generation time and tetraploid nature Xenopus Laevis is not suitable for genetics experiments. This disadvantage has been compensated with the introduction of morpholino based knock-down techniques (Eisen and Smith, 2008).

1.4.1 Overview of Early Development in Xenopus Laevis

The Xenopus oocyte is radially symmetrical and consists of a pigmented animal and an unpigmented vegetal hemisphere. The animal hemisphere contains the egg nucleus and receptors for sperm attachment. Fertilization activates microtubule polymerization which results in rotational movement of cortical cytoplasm away from the sperm entry point. This cortical rotation causes maternal determinants to be concentrated on one side of the vegetal hemisphere and set the dorsoventral axis, tissues furthest from sperm entry point being the dorsal (Weaver and Kimmelman, 2004). It also leads to an asymmetrical distribution of pigments making the dorsal side recognizable by lighter pigmentation.The first cell cycle lasts 90 minutes, and ends with the male and female pronuclear fusion. The next eleven divisions occur at 20 to 30 minute intervals with no gap phases forming a ball of about 4000 cells which encloses a fluid-filled blastocoel cavity. This mid-blastula embryo has three regions; the animal cap, marginal zone, and vegetal mass, that are already determined to form the three primary cell layers. Explants from animal caps form ectoderm, while marginal zone explants form mesoderm, and vegetal explants form endoderm. After twelfth cell cycle, cell divisions slow down, and zygotic transcription starts. This phenomenon is called mid-blastula transition, MBT.

In the fifteenth cycle, the dorsal lip of the blastopore forms, and gastrulation movements begin. Gastrulation movements convert the embryonic ball of cells into the three tissue layers, ectoderm, mesoderm, and endoderm, and establishes definitive anteroposterior and dorsoventral axes. In the anterior-posterior (AP) axis they form head, trunk, and tail. In dorsal-ventral axis (DV), the ectoderm (dorsal to ventral) gives rise to central nervous system, neural crest, cement gland, and epidermis. The mesoderm (dorsal

to ventral) gives rise to head mesoderm, notochord, somites, kidneys, heart, and blood. And the endoderm gives rise to digestive and respiratory systems (Heasman 2006) (Figure 1.2)

1.4.2 Role of Maternal Determinants

In Xenopus development**,** maternal stores of mRNAs and proteins are essential for embryonic patterning, because zygotic transcription does not start until 4000-cell stage. The localized positioning of maternal mRNAs provides the initial asymmetry for development. For example, transcripts of the transcription factors Zic2 and Xgrhl1 are localized to animal hemisphere, whereas mRNAs for VegT, Vg1, and Wnt11 are localized to vegetal hemisphere in the oocyte, and later in the embryo (Houston and Wylie, 2005; Tao et al., 2005; Zhang and King, 1996; Weeks and Melton, 1987; Ku and Melton, 1993). Both localized mRNAs themselves such as VegT, and cortical cytokeratin filament network are involved in the establishment of this initial asymmetry (Heasman, 2006).

The second asymmetry is provided by fertilization and resulting cortical rotation of the cytoplasm. Cortical rotation leads to the activation of canonical Wnt pathway, which results in the accumulation and nuclear localization of β-catenin at the dorsal side of the embryo (Larabell et al., 1997). Loss of function experiments suggested that Wnt11 is the main ligand inducing β-catenin activation in the dorsal side. (Tao et al., 2005) Despite large-scale transcription is being inhibited until mid blastula transition (MBT), Wnt signaling is required for Xnr5 and Xnr6 mRNA expression as early as 256-cell stage (Yang et al., 2002).

Other signaling pathways that are important for early patterning; TGFβ, BMP, and FGF has been shown to be inactive prior to MBT (Schohl and Fagotto, 2002). Therefore, Wnt/ β-catenin signaling is the only known signaling pathway that is active before the onset of large scale zygotic transcription and sets dorsal ventral axis.

1.4.3 From MBT to Gastrulation: Induction of Germ Layers

After the embryo reaches about 4000-cell stage, cell division slow down and zygotic transcription starts. The initial induction of zygotic genes is regulated by a combination of maternal factors. However, the newly synthesized molecules rapidly join the picture and working together with the maternal factors, form a complex network of signaling molecules. The activities of four signaling pathways; TGFβ , Wnt, FGF and BMP pathways are well characterized in the inductive and patterning events leading to the formation of the three primary germ layers, ectoderm, mesoderm, and endoderm.

Mes-endoderm Induction

Since mesoderm and endoderm is induced largely by the same molecules, I will cover signaling pathways and transcription factors involved in mes-endoderm induction together. Three maternal factors VegT, β-catenin, and Vg-1 are involved in mesendodermal induction. VegT, a vegetally localized transcription factor induces pro-endodermal transcription factors (mix.1, bix4, sox17, and endodermin) and TGFβ ligands; Xnr (Xenopus Nodal related) 1,2,4,5, 6, and derriere (Zhang et al., 1998; Xanthos et al., 2001; White et al., 2002; Taverner et al., 2005). β-catenin induces Xnr5

and 6, and enhances expression of other Xnrs in the dorsal side (Takahashi et al., 2000; Rex et al., 2002). Combined action of VegT and β-catenin ensures high levels of Xnr expression in the dorsal vegetal cells. Expression of Xnrs and derriere are reinforced by Xnr signaling itself through a positive feedback loop. This creates a dorsal ventral gradient of Xnr signaling with higher levels on the dorsal side which leads to the establishment of the Niewkoop center in the dorsal-vegetal cells, a specialized tissue which secretes high levels of Xnrs and Cerberus, an inhibitor of TGFβ, Wnt, and BMP signaling that is required for dorsoanterior development (Piccolo et al., 1999). Xnr signaling in the vegetal cell mass then induce and pattern endoderm together with pro-endodermal transcription factors induced by VegT. Xnr ligands also diffuse to the overlying marginal zone cells and induce dorsal mesoderm, which will then form the Spemann Organizer in the dorsal marginal zone, whereas the Nieuwkoop center cells themselves form anterior endoderm (Agius et al., 2000; Takahashi et al., 2000). A third maternal factor Vg-1, a TGFβ family ligand is also enriched maternally in the dorsal vegetal cells and is required for the dorsal expression of Cerberus, Noggin, and Chordin (Birsoy et al., 2006; Lebreton and Jones, 2006). There is also evidence that another TGFβ family member, activin B, is essential for normal development, and regulates dorsal mesendodermal genes goosecoid, chordin, and Xhex as well as TGFβ ligands Xnr2, and derriere (Piepenburg et al., 2004). High levels of TGFβ ligands induce the expression of ligands of another major signaling pathway, FGF (fibroblast growth family). FGF signaling is important for the formation of mesoderm in vertebrates, and when it is disrupted, most trunk and tail mesoderm fails to form (Amaya et al., 1993). In Xenopus explants, FGF signaling through MAPK is necessary for most mesoderm induction by

activin, and in vivo, multiple FGF ligands are involved in regulating mesoderm formation, including FGF4 and FGF8, which are necessary for paraxial mesoderm formation (Cornell and Kimelman, 1994; LaBonne et al., 1995; Fletcher et al., 2006; Isaacs et al., 2007). Temporal analysis of FGF signaling by using a chemical inhibitor revealed that FGF signaling is essential for the initial specification of paraxial mesoderm but dispensable for activation of several pan-mesodermal and most organizer genes. However, early FGF signaling is necessary for the maintenance of organizer gene expression into the neurula stage (Fletcher and Harland, 2008).

In conclusion, the combination of maternal factors VegT, β-catenin, and to some extent Vg1, generates a dorsal ventral gradient of TGFβ ligands in the vegetal cells that in turn activate transcription of a network of mesendodermal genes (Figure 1.3).

Dorsal Ventral Patterning and the role of BMP signaling

Genetic screens in Zebrafish and Drosophila have isolated numerous mutations that affect dorsal ventral patterning of the embryo. Most of these mutations affect Bone morphogenic protein (BMP) signaling pathway. Zebrafish mutations include swirl (BMP2b), snailhouse (BMP7) mutations that affect BMP ligands, chordino (Chordin), mini fin (Tolloid) mutations that affect extracellular availability of the ligand, lost-a-fin mutation that inactivates BMP receptor, and somitobun mutation which results in a dominant negative form of Smad5 trancription factor (Hammerschmidt and Mullins, 2002). BMP signaling in Xenopus is initially activated at MBT throughout the embryo except the dorsal animal quadrant (Schohl and Fagotto, 2002). Dorsal β-catenin signal which induces Nieuwkoop center in the vegetal cells is also present in the dorsal animal

cells. In this quadrant, β-catenin signal induces expression of BMP antagonists Chordin and Noggin (Wessely, 2001). This region is called Blastula Chordin- and Noggin-expressing (BCNE) center, is essential for the induction of neuroectoderm in blastula stage, and in later development, BCNE cells give rise to all of the forebrain, most of the mid and hind-brain, floor plate, and notochord (Kuroda et al., 2004). In addition to Chordin and Noggin, β-catenin signal in the dorsal animal tissue also induces Xnr3, a Nodal related protein without mesoderm inducing activity (Hansen et al., 1997). Interestingly, Xnr3 induces neural differentiation by binding to BMP ligands and acting as a competitive inhibitor through its pro-region (Haramoto et al., 2004). Thus, dorsal β-catenin signal induces two specialized regions that secrete antagonists of BMP signaling; the BCNE Center in the dorsal animal quadrant which secretes Chordin, Noggin, and Xnr3, and the Nieuwkoop Center in the dorsal vegetal quadrant, which secretes Cerberus, a combined inhibitor of TGFβ, Wnt, and BMP signaling. The expression of Chordin, and Noggin in BCNE is transient. At the onset of gastrulation, BCNE center and Nieuwkoop center is replaced by the Spemann Organizer and the same genes are re-expressed under the control of Nodal-related signals from Spemann Organizer (De Robertis and Kuroda, 2004). Nodal-related signals emanating from Spemann Organizer also induce FGF signaling ligands, which in turn activate Ras/MAPK signaling cascade. FGF signaling provides another level of negative regulation of BMP signaling in the dorsal side of the animal cells through phosphorylation of the linker region of Smad1 transcription factor (Kuroda et al., 2005). The linker phorphorylation acts as a tag for Smurf1 catalyzed polyubiqitination and subsequent proteosomic degradation of Smad1 (Zhu et al., 1999; Sapkota et al, 2007). Altogether, the cocktail of extracellular inhibitors of BMP signaling,

and inhibitory phosphorylation via MAPK signaling at Smad level restricts BMP signaling to the ventral side of the embryo (Figure 1.3).

In the ventral region of the embryo, BMP signaling acts to induce epidermal differentiation in the ventral animal cells, and impose a ventral character to the mesoderm in the marginal zone cells. In ventral animal tissues, BMP signaling induces two transcription factors; Xvent2 and Msx1, which activate the epidermal differentiation and also suppress pro-neural genes (Onichtchouk et al., 1996; Suzuki et al, 1997). Xvent2 and Msx-1 regulate pro-epidermal genes Xap2 (Feledy et al., 1999; Luo et al., 2002), Dlx3 (Luo et al., 2001), and Xgrhl1 (Tao et al., 2005), which in turn induce epidermal structural genes such as cytokeratin XK81A1 (Jonas et al., 1989) and X-epilectin (Masse et al., 2004).

In the ventral marginal cells, BMP signaling pathway imposes a ventral fate to the mesoderm. Overexpression of BMP ligands BMP2, 4, or7 or Smad1/5 transcription factor leads to the expansion of ventral mesodermal tissue, such as blood islands at the expanse of dorsal mesodermal tissues in a dose dependent manner. Inversely, a dominant-negative BMP receptor dorsalizes ventral mesoderm dose dependently from blood, to pronephros, to muscle, to notochord (Jones et al., 1996; Dosch et al., 1997; Hemmati-Brivanlou and Thomsen, 1995; Thomsen, 1996; Dale and Jones, 1999). The ventralizing activity of BMP signaling is largely through antagonism of Organizer signals at midblastula stage (Marom et al., 2005). This anti-organizer activity of BMP signaling is accomplished primarily by two homebox transcription factors Vent1 and Vent2 which inhibit Goosecoid expression outside of the Spemann Organizer (Gawantka et al., 1995; Onichtchouk et al., 1996; Melby et al., 2000; Imai et al., 2001). Loss-of-function

experiments indicate that Vent and Goosecoid transcription factors regulate each others function and form a self-adjusting mechanism that restores the basic body plan when deviations from the norm occur (Sander et al, 2007) (Figure 1.4).

Neural Induction

Xenopus ectoderm (animal cap tissue) differentiates into epidermis when explanted and cultured alone. However, when dissociated and are devoid of any cell-to-cell contact that can relay signals between neighboring cells, ectodermal cells acquire neural fate (Godsave and Slack, 1989). This neutralization can be reversed by addition of BMP4 to the culture medium, which led to the proposal that during dissociation BMP4 protein is diluted by diffusion into the culture medium (Wilson and Hemmati-Brivanlou, 1995). These findings led to the proposal of the "Default Model" which states that ectodermal cells are determined to form neural tissue unless instructed to form epidermis by BMP signaling (Hemmati-Brivanlou and Melton, 1997; Munoz-Sanjuan and Brivanlou, 2002). Over the last decades several other studies provided evidence for the "Default Model". First, over-expression studies showed that addition of BMP2/4/7 ligands, constitutively active BMP type I receptors, Smad1, and a downstream effectors of BMP signaling Msx1 transcription factor can induce epidermal differentiation in dissociated cells (Wilson and Hemmati-Brivanlou, 1995; Suzuki et al., 1997). On the other hand, inhibition of BMP signaling by various reagents including dominant negative receptors and dominant negative forms of downstream effectors Vent transcription factors induces neural tissues (rev. in; Harland, 2000; De Robertis and Kuroda, 2004, Stern, 2005). Second, The Spemann Organizer has been known for its neural inducing

capacity when transplanted to the ectoderm of a host embryo (Spemann and Mangold, 1923 (reprint in 2001)). The activity of Spemann Organizer is carried out by a cocktail of secreted molecules; Noggin, Chordin, Follistatin, that inhibit BMP signaling by binding to BMP ligands and sequestering them from binding to their receptors. (Hemmati-Brivanlou and Melton, 1997; Sasai and De Robertis, 1997) When Chordin, Noggin, and Follistatin are knocked-down at the same time, the embryos lose all dorsal mesodermal tissues and do not form neural plates (Khokha et al., 2005). On the other hand, knock-down of all four BMP ligands, BMP2, 4, 7, and ADMP, that is present in early Xenopus development, causes ubiquitous neural induction throughout the ectoderm (Reversade and De Robertis, 2005). In a related study, triple knock-down of BMP2, 4, and 7 and elimination of the Spemann organizer by UV treatment or β-catenin depletion led to the loss of all ventral development and resulting in embryos having radial central nervous system (CNS) structures (Reversade et al., 2005b). Therefore, BMP repression is sufficient for neural induction in vivo, and that in the absence of ventral BMPs, Spemann organizer signals are not required for brain formation.

Although there is overwhelming evidence that supports the default neural induction model in Xenopus, studies in Zebrafish and Chick embryos has pointed out a role for FGF and Wnt signaling (Wilson and Edlund, 2001; Stern, 2002; Lemaire et al., 2002). As previously discussed, FGF ligands relay their signal through activation of ras/MAPK cascade, which is shown to inhibit BMP signaling by facilitating degradation of Smad1 transcription factor (Kuroda et al., 2005). This is consistent with the default model as the mechanism is another strategy for inhibition of BMP signaling in prospective neuroectoderm. However, in Zebrafish the topology of neural induction is

different from Xenopus and the tissues spinal cord arise, extent far from the Organizer to the ventral side of gastrula. In these tissues, FGF signaling is required to induce vegetal prospective neural markers without suppressing Bmp signaling (Kudoh et al., 2004; Rentzsch et al., 2004). This study shares similarity with an earlier work in Xenopus which showed that FGF signaling is critical for the formation of posterior neural tissues but is dispensable for neural induction (Ribisi et al., 2000).

When BMP signaling is inhibited in ventral ectoderm cells of Xenopus, this does not lead to induction of neural markers in intact embryos (Linker and Stern, 2004; Delaune et al., 2005). This detail has raised some eyebrows among the critics of the default neural induction model. Neural induction is achieved only when BMP inhibition is combined with FGF addition. Consequently, when FGFR activity is blocked by chemical inhibitor in blastula stage embryos, inhibition of BMP signaling is not enough to restore neural tissues (Delaune et al., 2005). Therefore, it is suggested that FGF signaling has neural inducing activity independent of its inhibitory effect on BMP signaling at Smad level. However, a recent paper by Harland lab provides a different explanation. Manipulations that inhibit TGFβ/Smad2 pathway and BMP/Smad1 pathway at the same time induce early neural markers and inhibit epidermal genes in ventral ectoderm (Chang and Harland, 2007). They proposed that in addition to blocking Smad1, FGF/MAPK signaling may also inhibit Smad2 through linker phosphorylation, and this may contribute to the synergistic effect on neural induction by BMP inhibitors and low FGF signaling in previous studies (Linker and Stern, 2004; Delaune, 2005). In agreement with this model, inactivation of Smad2 by linker phosphorylation has been correlated with loss of competence of gastrula ectoderm to respond to activin-mediated mesodermal

induction (Grimm and Gurdon, 2002). Additional mechanisms exists such degradation of Smad4 by ectodermin, which results in the reduction of both BMP and nodal type TGFβ signaling dorsal animal quadrant, where the abrogation of BMP and Nodal signaling is required for neural specification (Dupont et al., 2005). Wnt signaling has also been implicated in neural induction. Both in chick and Xenopus development, Wnt inhibition cooperates with FGF to induce neural fates (Wilson et al., 2001; Heeg-Truesdell and LaBonne, 2006). It appears that Wnt signaling acts in a similar manner to FGF signaling through regulating the duration of BMP signal at Smad1 transcription factor level. In this case, Wnt signaling acts to inhibit GSK3β catalyzed linker phosphorylation of Smad1, and redistributes Smad1 from centrosomes to cytoplasmic LRP6-signalasomes (Fuentealba et al., 2007).

In conclusion, overwhelming evidence suggests that neural induction of the (anterior regions) central nervous system is mediated by BMP inhibition alone, while neural induction of (posterior regions) spinal cord is mediated by a combination of FGF signaling and BMP signaling (Figure 1.3 and Figure 1.5).

Onset of Gastrulation; Opposing Forces are ready to march on.

By the start of gastrulation, combinatorial actions of Wnt , Nodal type TGFβ, BMP ,and FGF pathways have already led to the formation of defined territories for future body plan. In the dorsal vegetal side, actions of maternal maternal factors VegT, Vg1 and β-catenin induced the Nieuwkoop center. Nieuwkoop center cells express Nodal related TGFβ ligands, head inducer Cerberus, and Wnt signaling inhibitors; Dkk-1, Crescent, and Frzb-1 (Agius et al., 2000; Takahashi et al., 2000; Bouwmeester et al.,

1996; Glinka et al., 1998; Pera and De Robertis, 2000; Silva et al., 2003). This combination forms a BMP, WNT and FGF signaling free zone with limited Nodal/TGFβ activity, which is essential for the formation of dorsal anterior endoderm (head induction). VegT and Nodal/ TGFβ activity in the vegetal cells induce and pattern the endoderm by activating the expression of endodermal transcription factors; mix.1, mixer, bix4, sox17α/β, sox7, endodermin, and GATA 4-6 (Wardle and Smith, 2006). At the onset of gastrulation, Nieuwkoop center induces Spemann Organizer, in the overlying marginal cells. Spemann Organizer quickly takes over the role of Nieuwkoop center as a source of secreted Nodal type TGFβ ligands and induces and patterns mesoderm in the marginal cells (Kuroda and De Robertis, 2004). The Spemann Organizer itself becomes dorsal axial mesoderm (notochord) and induces neighboring cells to adopt other dorsal fates, such as neural plate and paraxial mesoderm. In addition to being a source of Nodal Related TGFβ signals, Organizer also secretes a cocktail of inhibitors. The first group includes BMP signaling inhibitors; Chordin, Noggin, and Follistatin, and Xnr3 (Piccolo et al., 1996; Zimmerman et al., 1996; Iemura et al., 1998; Haramoto et al., 2004). These inhibitors were also expressed earlier at late blastula stage in response to maternal β-catenin activity in dorsal animal quadrant (Kuroda and De Robertis, 2005). The expression of these inhibitors in BCNE at blastula and in Spemann Organizer at gastrula ensures continued suppression of BMP activity in the dorsal side of the embryo, which leads to neural induction in ectodermal cells adjacent to Spemann Organizer and establishment of dorsal mesoderm (Figure 1.4) The second group includes Wnt signaling antagonists; Frzb-1, Crescent, sFRP-2, and Dickkopf (Kavano and Kypta, 2003). Although initial β-catenin activity is required for induction of Spemann Organizer,

induction of head mesoderm and notochord requires inhibition of Wnt signaling (Glinka et al., 1998; Schneider and Mercola, 2001; Shibata et al., 2005).

Inhibition of Wnt signaling is also required for neural induction in the adjacent ectoderm (Heeg-Truesdell and LaBonne, 2006). Actions of Nodal related TGFβ signaling, FGF signaling and inhibition of Wnt and BMP signaling in Spemann Organizer collectively results in expression of transcription factors that are involved in convergent extension and gastrulation movements (such as Xlim1, Xotx2, Goosecoid, Xbra) and myogenesis (such Xbra, MyoD, Myf5) (Bouwmeester, 2001; Heasman, 2006).

While Spemann Organizer forms the primary signaling center of the Xenopus embryo in the dorsal side, sustained BMP signals form a secondary signaling center in the ventral side form the "Ventral Gastrula Center"(VGC) (Kuroda and De Robertis, 2004). BMP signaling in the ventral side counteracts dorsalizing signals from Spemann Organizer by inducing its own set of antagonist and transcription factors. Two of the antagonist secreted by VGC; Twisted-gastrulation and Tolloid are involved in inactivation of Chordin (Oelgeschlager et al., 2000; Piccolo et al., 1997). In addition to antagonists, BMP induced transcriptional factors Msx-1, Vent1 and Vent2 counteract Organizer function by inhibiting expression of organizer genes outside of the Organizer domain (Figure 1.4) (Takeda et al., 2000; Sander et al., 2007). BMP signaling in the mesoderm is critical for patterning of mesoderm into lateral and ventral mesoderm. BMP regulated induction of Vent1 and GATA-2 regulates blood development at the ventral most mesoderm where BMP signaling is highest (Xu et al., 1999; Schmerer and Evans, 2003). In ventral lateral mesoderm BMP signaling has moderate levels due to antagonists secreted from Organizer, and is involved in patterning of ventrolateral genes Xpo, Wnt8,

Myf5, and Tbx6 (Sato and Sargent, 1991; Marom et al., 1999;Marom et al., 2005; Szeto and Kimelman, 2004). Both the Spemann Organizer and the Ventral Gastrula Center act as self regulating morphogenic fields by expressing antagonist against their own activities, such ADMP, a BMP type ligand in Spemann Organizer, and BAMBI, a truncated BMP receptor in the VGC (Moos et al., 1995; Dosch et al., 2000; Onichtchouk et al., 1999) (Figure 1.4).

Gastrulation movements convert the embryo into the three tissue layers, ectoderm, mesoderm, and endoderm, and establishes definitive anteroposterior and dorsoventral axes. In the anterior-posterior (AP) axis they form head, trunk, and tail. In dorsal-ventral axis (DV), the ectoderm (dorsal to ventral) gives rise to central nervous system (low BMP, low FGF signaling), neural crest, cement gland, and epidermis (high BMP signaling). The mesoderm (dorsal to ventral) gives rise to head mesoderm (high Nodal), notochord, somites, kidneys, heart, and blood (high BMP). And the endoderm gives rise to digestive and respiratory systems. (Figure 1.4 and 1.5).

1.5 Figures

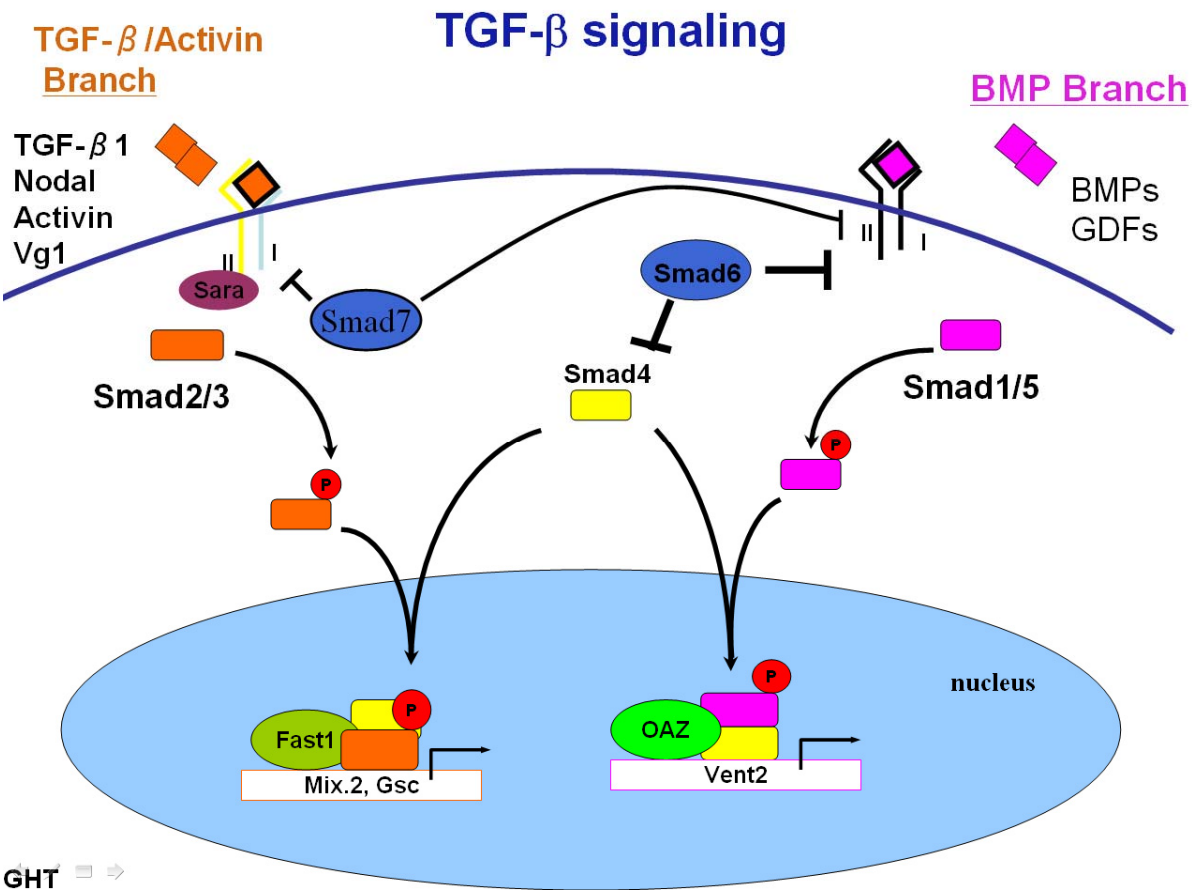

Figure 1.1: TGF-β signalling pathways in early Xenopus development. The BMP pathway is shown on the left and the Activin/Nodal signalling pathway is on the right. Receptor activation leads to phosphorylation and activation of R-Smads, which are XSmad1 and XSmad5 for the BMP receptors and XSmad2 and XSmad3 for the Activin/Nodal receptors. The R-Smads then form complexes with co-Smads, XSmad4α and XSmad4β. Activated Smad complexes are recruited to DNA via specific transcription factors such as xOAZ for Smad1/Smad4 complexes and xFast-1 or Mixer for Smad2/Smad4 complexes. The pathway is further regulated at different points as shown. Ligand antagonists function extracellularly, probably preventing ligand binding to the receptor. Inhibitory Smads (xSmad6 and xSmad7) can act at the level of the receptors or in the case of Smad6, can also compete with Smad4 for activated Smad1 (modified from Gerald H. Thomsen)

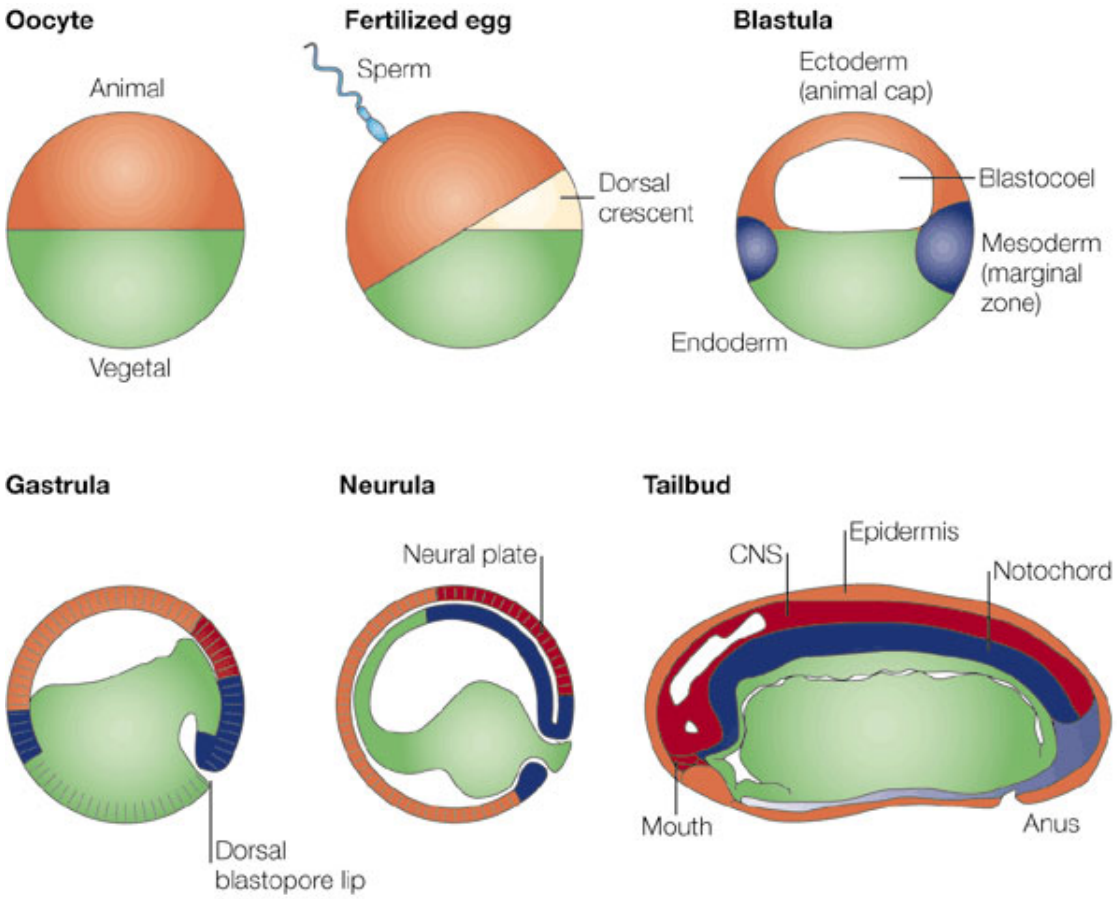

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Figure 1.2: Overview of Xenopus development.

The Xenopus oocyte is radially symmetrical and is divided into an animal and a vegetal halves. One hour after fertilization, an unpigmented dorsal crescent is formed in the fertilized egg opposite the sperm entry point. As the embryo rapidly divides into smaller and smaller cells, without intervening growth (cleavage), a cavity called the blastocoel is formed, which defines the blastula stage. By the late blastula stage, the three germ layers become defined. The ectoderm, or animal cap, forms the roof of the blastocoel. The mesoderm is formed in a ring of cells in the marginal zone, located between the ectoderm and endoderm. At the gastrula stage, involution of the mesoderm towards the inside of the embryo starts at the dorsal blastopore lip. The morphogenetic movements of gastrulation lead to the formation of the vertebrate body plan, patterning the ectoderm, mesoderm and endoderm. At the neurula stage (14 h), the neural plate, or future central nervous system (CNS), becomes visible in dorsal ectoderm. By the tailbud stage (24–42 h), a larva with a neural tube located between the epidermis and the notochord has formed. The blastopore gives rise to the anus, and the mouth is generated by secondary perforation (From De Robertis et al., 2000).

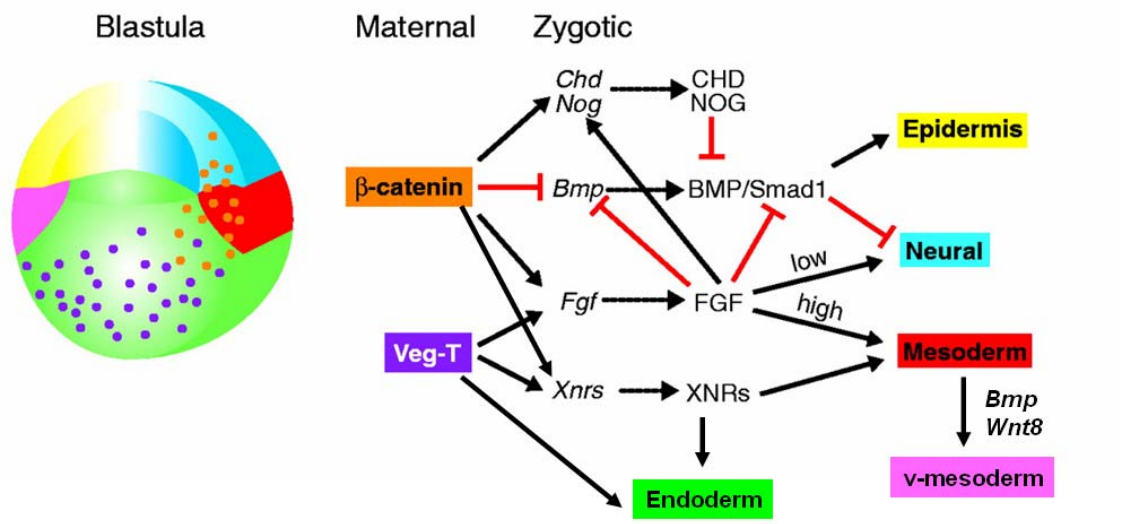


Figure 1.3: Induction of the three primary germ layers. In early blastula stage, there is no zygotic transcription. Maternal transcription factor VegT is localized to the vegetal half, whereas β-catenin is stabilized and localized to the nuclei on the dorsal side. These two transcription factors induce Xnrs (Xenopus Nodal Related Ligands), and FGFs. β-catenin antagonizes BMP signaling by inducing soluble inhibitors of BMP signaling and indirectly by inducing FGF signaling. In late blastula, endoderm is formed in vegetal cells under influences from VegT and Xnrs. Xnrs and high FGF signaling in equatorial cells specify mesoderm. BMP signaling is active in ventral ectoderm, and specify epidermis, whereas in dorsal ectoderm, BMP inhibitors and low FGF signals specify neural tissues. BMP together with Wnt8 act to ventralize mesoderm in the ventral side of the embryo (modified from Stern, 2005, and Delaune et al., 2005).

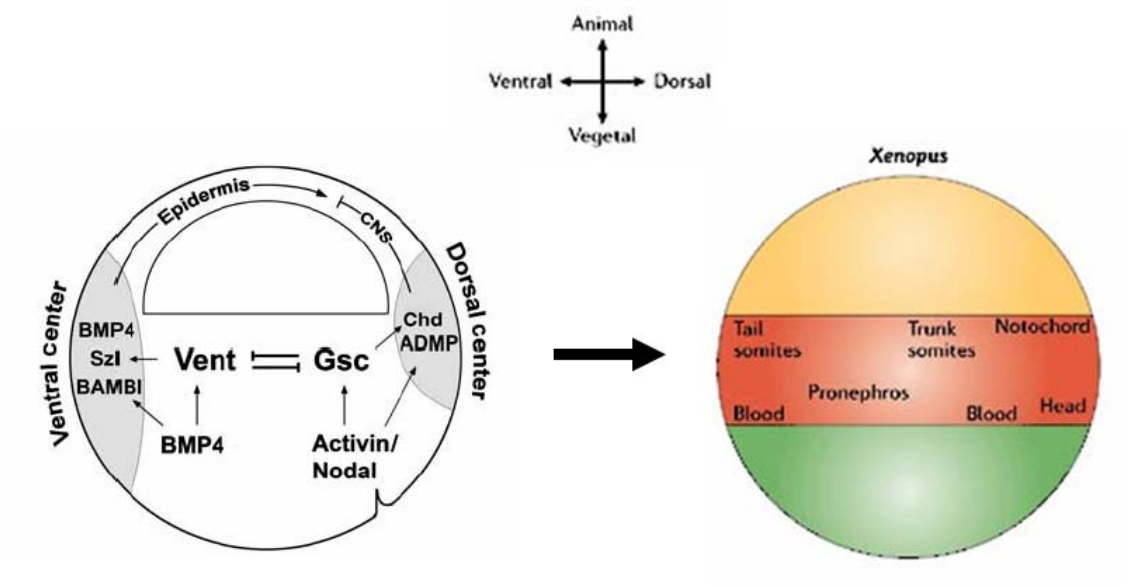


Figure 1.4: Dorsal-ventral patterning of mesoderm by Xnrs and BMP signaling. Activin/Nodal signaling and inhibitors of BMP in the dorsal side leads to the formation of Spemann Organizer (Dorsal Center), whereas in ventral side high BMP signaling results in the formation of a Ventral Center. BMP signaling induces Vent1 transcriptional repressor in ventral mesoderm/ventral ectoderm, and Xnrs and β-catenin induce Goosecoid transcriptional repressor in the Spemann Organizer. Vent2 and Gsc counteract each other activities, resulting in patterning of mesoderm, blood being induced at highest levels of BMP, and head and notochord being induced where Xnrs are highest (modified from Sander et al., 2007; Kimelman, 2006).

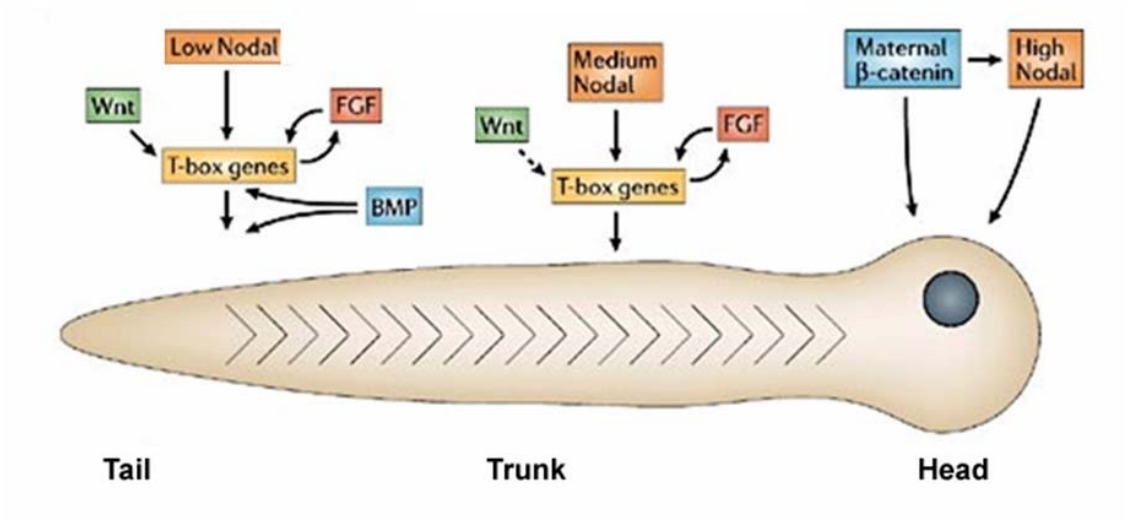


Figure 1.5: Axial patterning of Xenopus embryos by combined action of Nodal, Wnt, FGF, and BMP signaling. (modified from Kimelman, 2006)

Chapter 2. Isolation and Characterization of GTPBP2 as a Smad binding protein

2.1 Introduction

The Transforming Growth Factor β (TGF-β) superfamily of signaling proteins regulate a diverse set of biological processes, including cell proliferation, adhesion, migration, apoptosis, differentiation and embryonic pattern formation (Shi and Massague, 2003; ten Dijke et al., 2002; Hill, 2001; Schier and Talbot, 2005).

Because of its importance in embryonic development and tissue homeostasis, a variety of mechanisms are evolved to regulate the boundary, intensity, duration and activity of TGF-β pathway. These mechanisms include use of competitive inhibitors in the extracellular matrix that are limiting ligand accessibility to the receptor (Noggin, Chordin), truncated receptor homologs (BAMBI) at the cell membrane, inhibitory Smads (Smad6-7), sequestering (MAN1) and, or degradation (Smurf1, Ectodermin) of Smad proteins within nucleus and cytoplasm (Piccolo et al., 1996; Zimmerman et al.,1996; Onichtchouk et al., 1999; Osada et al., 2003; Zhu et al, 1999; Dupont et al., 2005; Itoh, and Dijke 2007).

To identify novel molecules that regulate the intracellular BMP/Smad1 signaling pathway, a former graduate student, Haitao Zhu, did a yeast two-hybrid screen using Smad1 as bait, and retrieved GTPBP2 as a binding partner for Smad1 (Zhu et al. 1999). GTPBP2 and its close homolog GTPBP1 are large GTPases of unknown function. GTPBP2 was first isolated through its interaction with cytoplasmic tail of BH-Protocadherin-C, and by its homology to GTPBP1 (Watanabe et al., 2000; Senju et al.,

2000). These two studies are limited in scope to characterization of gene structure and expression analysis. In a third study, translocations involving GTPBP2 is linked to glioblastoma formation (Mulholland et al., 2006).

In this chapter, I will describe the biochemical experiments that provided us basis for biological and embryological assays. Here, I show GTPBP2 interacts with Smad1 in vitro, and in vivo GTPBP2 co-immunoprecipitates with Smad1, 3, 4, and 6. This interaction is mediated primarily through MH1 domain of Smad1 and C-terminus of GTPBP2. GTPBP2B is a nuclear protein and co-localizes with Smad1 to nuclear foci.

2.2 Results

2.2.1 Identification and Sequence Analysis of GTBPP2

To identify potential new regulators of Smad signaling, a former graduate student in the lab, Haitao Zhu performed a yeast two hybrid screen using Xenopus Smad1 as bait to probe an oocyte cDNA library (Zhu et al., 1999). One of the clones retrieved encodes the C-terminal 144 amino acids of Xenopus homolog of GTPBP2, a large GTPase that together with a homolog named GTPBP1, define a small but distinct subclass of the GTPases with homology to translation elongation factor eEF1A and its paralogs (22% identity, 39% similarity). Orthologous genes to *Gtpbp2* are found throughout Bilateria.

I used the partial-length Y2H clone and GTPBP2 sequence information to retrieve *Xenopus laevis* ESTs that encode two slightly different proteins which differ at their N-termini (Figure 2.1). One set of predicted GTPBP2 transcripts encodes a long form of the protein which I term GTPBP2A, and the other set encodes a shorter form, GTPBP2B,

whose initiation methionine (Met) codon and downstream sequences are contained within the open reading frame (ORF) of GTPBP2A. Previously published papers suggests different start sites for GTPBP2 corresponding to a 514aa long human protein, and a 584 a.a. long mouse homolog (Senju et al., 2000; Kudo et al., 2000). In addition to this, human reference sequence at NCBI database (NM_019096) suggests a third start site upstream of the ones described in papers mentioned above, which translates into a human protein of 602aa length.

To clarify the origin and authenticity of these transcripts and their encoded variant proteins, I compared the human GTPBP2 genomic locus to human cDNAs. This analysis revealed that human GTPBP2A and GTPBP2B forms result from alternative splicing that, respectively, incorporated either Exon1a or Exon1b, (Figure 2.2) into the mature transcript. Hence, I concluded that human *Gtpbp2* locus codes for two forms of GTPBP2 protein. There exists some ambiguity in defining the N-terminus of GTPBP2A in X. laevis and X. tropicalis, due to two in-frame Met codons within the first 20 aa of the longest ORF. The first of these Met codons precedes a polypeptide sequence found only in X. laevis and X. tropicalis, and I term this putative form GTPBP2A$_L$. The initiation Met and flanking peptide sequence of the GTPBP2A N-terminus occurs 19 aminoacids internal to the N-terminus of GTPBP2A$_L$, and this internal site is conserved and aligns with the N-termini of GTPBP2A from other species (Figure 2.3). The start codons of Xenopus GTPBP2A and GTPBP2B transcripts are preceded by good Kozak consensus sequences, but the start codon of GTPBP2A$_L$ lacks an adjacent Kozak consensus, suggesting that GTPBP2A$_L$ is less likely to be translated, if at all. I have taken this detail into consideration when designing morpholinos for loss-of-function experiments.

2.2.2. GTPBP2 interacts with Smad1 in vitro

To confirm interaction between Smad1 and GTPBP2, I performed an in vitro interaction assay using a GST-xSmad1 fusion protein. I purified GST-xSmad1 and GST proteins from bacteria and produced radioactively labeled GTPBP1 and GTPBP2 proteins using rabbit reticulocyte system. I used GST protein as a negative control and xSmurf1 as a positive control for interaction assays. In vitro translated GTPBP2 protein interacted with Smad1 in GST pull-down assay. (Figure 2.4) On the other hand, I did not observe an interaction between GST-Smad1 and GTPBP1. GST protein controls did not have any background and Smurf1, a well characterized binding partner, was also successfully precipitated with by GST-xSmad1.

2.2.3 GTPBP2 interacts with Smads in cell culture

To verify this in vitro interaction, and to determine whether GTPBP2 binds to any other Smads, I performed co-immunoprecipitation assays using transfected COS-1 cells over-expressing GTPBP2 and Smads. To assess the GTPBP2 range of binding specificity, I challenged the xGTPBP2B with the major R-Smads (Smad1, Smad2 and Smad3), co-Smad (Smad4), and I-Smads (Smad6 and 7) (Figure 2.5B). Flag-tagged versions of the Smads were immunoprecipitated and tested for binding with HA-GTPBP2 by Western Blot. I found that GTPBP2 can interact with Smads 1, 3, 4, and 6, in a very reproducible fashion (Figure 2.5B)**,** but interactions with Smad2 and Smad7 were either not observed, or were weak and not reproducible (data not shown).

2.2.3. Smad1 binds to conserved C-terminal motifs in GTPBP2 through its MH1 domain

I mapped the interacting domains of GTPBP2 and Smad1 by co-IP experiments on full-length or deletion mutant proteins expressed in COS-1 cells (Figure 2.5). Full-length and deletion mutants of GTPBP2B were tagged at the N-terminus with 3xHA epitope. GTPBP2 consists of a GTPase domain at the N terminus followed by two conserved domains at their C termini, named as GTPBP_II and GTPBP_III (Figure 2.5A). ΔN-GTPBP2B lacks the GTPase domain but retains conserved GTPBP_II and GTPBP_III motifs that are also present in the original yeast two hybrid clone, whereas ΔC-GTPBP2B codes for GTPase domain (Figure 2.5A).

Smad proteins have three conserved domains, the N-terminal Mad homology domain (MH1), a variable praline-rich linker domain, and the C-terminal MH2 domain. The MH2 domain is highly conserved among all Smads, is in R-Smads involved in type I receptor binding and becomes directly phosphorylated in its C-terminal SSXS motif by type I receptors (Heldin et al., 1997). In addition to receptor binding, MH2 domain is required for Smad oligomerization and Smad4 binding, and is shown to interact with cytoplasmic adapters (e.g. par3 and dishevelled-1) and various transcription factors (e.g. OAZ, Runx, and Znf8) (Warner et al., 2003; Hata et al., 2000; Jiao et al., 2002; Massague et al., 2005). The MH1 domain is conserved among R-Smads and Smad4 (partially conserved in I-Smads), interacts with several cytoplasmic proteins (calmodulin, filamin), and is required for nuclear import of Smads (Sasaki et al., 2001; Schmierer and Hill, 2005). The MH1 domain mediates R-Smad/Smad4 binding to DNA, and is shown to interact with several transcription factors (e.g.Vent2, and FoxO) (Shi et al., 1998;

Henningfeld et al., 2002; Seone et al., 2004). Variable linker region is shown to be a target of MAPK and GSK3 mediated phosphorylation events, which in turn trigger Smurf binding through PY motifs in linker region and subsequent proteosomal degradation of Smads (Sapkota et al., 2007; Fuentealba et al., 2007).

Full-length and deletion mutants of XSmad1 were tagged at the C-terminus with the Flag epitope. Clone Flag-MH1 lacks the linker and MH2 domains, and Flag-MH2 lacks the MH1 and linker domains. In co-IP experiments full-length Smad1 bound to full-length GTPBP2B and ΔNGTPBP2B, but not ΔCGTPBP2B. In the converse experiment, full-length GTPBP2B bound to full-length Smad1 and the MH1 domain at high affinity (Figure 2.5C and D).These results demonstrate that the C-terminus of GTPBP2 interacts primarily with the MH1 domain of Smad1. I have not yet mapped the detailed sites of this interaction.

2.2.4. GTPBP2B is a nuclear protein and co-localizes with Smad1 to nuclear foci

To address where GTPBP2 might function, I constructed epitope tagged versions of GTPBP2 and examined their location in cultured cells and dissociated Xenopus animal caps. In COS-1 cells, over-expressed HA-GTPBP2B localized to the nucleus in a punctate pattern (Figure 2.6A). When Smad1 was co-transfected with GTPBP2B they co-localized in the nucleus in a similar speckled pattern observed with GTPBP2B alone (Figure 2.6C-D). Smad1 itself localized throughout the cell with majority of cells showing cytoplasmic localization rather than nuclear (Figure 2.6B).

I also found that GTPBP2 localized to the nucleus in Hek293T cell lines, as well as dissociated Xenopus animal cap cells cultured on fibronectin coated slides (Figure 2.7,

2.8). There were some differences in the extent of nuclear localization between GTPBP2A and GTPBP2B forms in COS-7 and Hek293T cells, depending on GTPBP2 expression levels. For example, in Hek293-T cells, Cherry-GTPBP2B was predominantly nuclear (Figure 2.7A), whereas Cherry-GTPBP2A has a mainly cytoplasmic localization (Figure 2.7B). At high levels, GTPBP2A is localized to the nucleus as well as to cytoplasmic foci (Figure 2.7C).

2.3 Discussion

GTPBP2 interacts with Smad1, Smad3, Smad4, and Smad6. Smad1 is the principal transcription factor downstream of BMP ligands in early Xenopus development, and over-expression of Smad1 or BMP2/4/7 leads to expansion of ventral mesodermal tissues at the expanse of dorsal mesodermal tissue, upregulation of ventral genes (Vent1, Wnt8, Xhox3), and suppression of Organizer markers (Gsc, Xnot) (Jones et al., 1996; Dosch et al., 1997; Hemmati-Brivanlou and Thomsen, 1995; Thomsen, 1996; Dale and Jones, 1999). On the other hand, Smad6 inhibits BMP signaling by binding to activated BMP receptors and Smad4, and reducing their accessibility to Smad1, leads to embryo dorsalization, and partial axias duplication (Imamura et al., 1997; Hata et al., 1998; Goto et al., 2007 -miyazawa-). Smad6 also functions in the nucleus as a transcriptional repressor and antagonizes BMP signaling by recruiting transcriptional co-repressors to BMP responsive promoters (Bai, and Cao, 2002: Lin et al., 2003). Smad2 is the more abundant transcription factor downstream of Activin/Nodal signaling in Xenopus. However, Smad3 is also expressed maternally, and during gastrulation albeit at much lower levels (Howell et al., 2001). Overexpression of Smad2/Smad3 and Activin/Nodal

ligands induce expression of dorsal mesodermal markers in *Xenopus* explant assays, and lead to the formation of partial secondary body axes (Baker and Harland, 1996; Chen et al., 1997; Graff et al., 1996), and studies in mice has shown that both Smad2 and Smad3 are necessary for induction and patterning of mesoderm, as only *Smad2*; *Smad3* double homozygous mutants entirely lack mesoderm and fail to gastrulate (Dunn et al., 2004). Smad4, common Smad, is utilized by both Activin/Nodal and BMP branches of TGF-β signaling. Consequently, it is required for both signaling pathways in Xenopus (Chang et al., 2006). To sum up, GTPBP2 interacts with both BMP-specific (Smad1 and Smad6), and Activin/nodal specific (Smad3) Smads as well as common Smad, Smad4. Therefore, it is likely that GTPBP2 acts as a general co-factor for TGF-β signaling in various biological processes.

Gtpbp2 locus codes for two forms of GTPBP2 protein; GTPBP2A and GTPBP2B. Previous reports on the size of GTPBP1 and GTPBP2 proteins were inconsistent with reference sequence (NM_019096) at NCBI. I compared human genomic locus with available ESTs/mRNAs and concluded that human GTPBP2 locus codes for a shorter form of GTPBP2 (GTPBP2B) protein that is missing first 78aa's, in addition to the evolutionary conserved form GTPBP2A. GTPBP2B form is previously described in mice (Watanabe et al., 2000). In Xenopus EST database, I found two ESTs coding for GTPBP2A (DT061674 and BF610978), and one for GTPBP2B (BI449029). Therefore, I concluded that GTPBP2 protein may exist as a shorter form in Xenopus as well. This had many implications for my analysis. GTPBP2B may be a decoy or dominant negative form of GTPBP2A. Or GTPBP2A and GTPBP2B may have different activation dynamics. For example, one of them may need a co-factor or signaling input for

activation. Additionally, they may have different in vivo roles. Therefore, I made constructs for over-expression and designed morpholinos for knock-down of both forms of the protein, GTPBP2A and GTPBP2B individually.

GTPBP2B is localized to the nucleus in a speckled pattern. Subcellular localization of GTPBP2 may give us an idea where and how GTPBP2 might function. I found that in cell lines, ectopic GTPBP2B is localized mainly to the nucleus, and in COS-7 cells, GTPBP2B has speckled nuclear pattern, with some cells showing localization to distinct foci. In addition, GTPBP2B recruited Smad1 to these nuclear foci. The localization of Smads to subnuclear foci resembling the speckled pattern I showed has been observed previously (Janknecht et al., 1998; Yoshida et al., 2000). In particular, activated Smads (Smad1 and 5) are directed to subnuclear foci of active transcription in the presence of Runx proteins (Zaidi et al., 2002). It is also suggested that Tob, an inhibitor of BMP signaling, directs Smads to nuclear foci as of negative regulation (Yoshida et al., 2000). Therefore, it is likely that these nuclear foci GTPBP2B and Smad1 co-localized to are sites of transcription or transcriptional regulation. In support of this, GTPBP2 interacts with Smad1 primarily through MH1 domain. The MH1 domain mediates R-Smad/Smad4 binding to DNA, and is shown to interact with several transcription factors (e.g.Vent2, and FoxO) (Shi et al., 1998; Henningfeld et al., 2002; Seone et al., 2004).

Interestingly, ectopic GTPBP2A has a more cytoplasmic localization in Hek293T and COS cells. However, it s localized to the nucleus as well as cytoplasmic granules at high levels of expression. In dissociated Xenopus embryonic cells, both GTPBP2A and GTPBP2B were mainly nuclear. These experiments suggest that subcellular localization

of GTPBP2A is regulated and requires a signal that is present in Xenopus cells but absent in COS and Hek293T cells. Since GTPBP2B is in the nucleus in all cells tested, the missing N-terminal aminaocids in GTBP2B are likely to be the site of subcellular regulation.

2.4 Materials and Methods

2.4.1 Isolation of GTPBP2 and constructs used in experiments

A partial clone of GTPBP2 was retrieved in a yeast two hybrid screen of Xenopus oocyte cDNA library (Clontech) using Smad1 as bait. I purchased cDNA clones of GTPBP2 coding for GTPBP2B (BI449029) and GTPBP2A (DT061674) from ResGen. GTPBP2A and GTPBP2B were then sub-cloned into pCS2-3xHA for co-immunoprecipitation and immunofluorescense experiments. Cherry-GTPBP2A and Cherry-GTPBP2B clones were constructed by replacing 3xHA tag in pCS2-HA with Cherry sequence.

pCS2-C'-Flag-Xmad1 was cloned by Haito Zhu. Flag-MH1 and Flag-MH2 were derived from this parental clone by PCR deletion of excluded sequences. ΔN-GTPBP2B and ΔC-GTPBP2B was subcloned in pCS2-3xHA by PCR. All PCRs were performed using Platinum Pfx polymerase (Invitrogen) with low cycle number (< 18 cycles). All constructs of Smad1 and GTPBP2 were cloned between XhoI and XbaI restriction sites in pCS2, pCS-3xHA, and pCS2-Cherry. GFP-Smad1 is a gift from Dr. John B. Gurdon.

2.4.2. GST pull-down assay

GST and GST-Smad1 proteins were expressed in BL21DE3 (Novagen) bacteria cells. One liter cultures were grown to 0.6 O.D. and induced with 1mM IPTG for 6 hours at RT. Bacteria were then lysed in 15ml PBS based lysis buffer containing 1% Triton-X 100, 10 mg/ml lysozyme, and Roche complete inhibitor tablet by sonication. Cleared lysated were incubated with GST beads for 4 hours at 4 C, and then washed with PBS containing 1% Triton-X five times. GST and GST-Smad1 were then eluted using 50mM imidazole. Imidazole was removed by dialysis. ^{35}S- labeled GTPBP1, GTPBP2, and Smurf1 proteins were translated from untagged parental cDNAs constructs using rabbit reticulocyte system (Promega). For GST-pull down, purified and labeled proteins were incubated for 2hours at 4 C, and then GST beads added for 1 more hour rotating at 4 C. The mixtures were precipitated by centrifugation at 3000 rpm and washed with PBS, repeated 4 times. The pellets were analyzed by SDS-PAGE/Western Blotting.

2.4.3 Immunoprecipitation and Immunofluorescense

Full-length and deletion constructs of HA-GTPBP2 were co-transfected with Flag-Smads and deletion constructs of Smad1 to COS-1 cells using transfection reagent Fugene6 (Roche). Cells were lysed 24 hours after transfection with PBS containing 1% Triton X-100, 2mM EDTA, 1mM Na_3VO_4 and complete protease inhibitors (Roche). To pull down Flag-Smads, anti-Flag M2 agarose (Sigma) beads were incubated with lysates for 1 hour at 4C. Beads were spun and washed with cold lysis buffer several times, and SDS sample buffer was added to the beads and proteins were resolved by SDS-PAGE.

Anti-HA-HRP (Roche) (1:500) and anti-Flag M2 (Sigma) (1:2000) followed by anti-mouse-conjugated HRP (Sigma) (1:5000) were used to detect HA-GTPBP2 forms and Flag-Smads, respectively.

For immunofluorescence assays, HA and Cherry tagged version of GTPBP2B and GTPBP2A were transfected with or without myc-Smad1 into COS and HEK293T cells. Over-expressed proteins were detected with a-myc 9E10 (tissue culture supernatant) (1-50 dilution), poly clonal anti-HA (ICL) (1-250 dilution) antibodies, which were detected with Alexa 488 goat anti-mouse and Alexa 594 goat anti-rabbit (Invitrogen). 1ng Cherry-GTPBP2A or GTPBP2B were injected into embryos at 2-cell. Animal caps were excised from these embryos, dissociated, and grown on fibronectin coated slides as described in (Simeoni and Gurdon, 2007) for visualization of Cherry tagged clones.

2.5 Figures

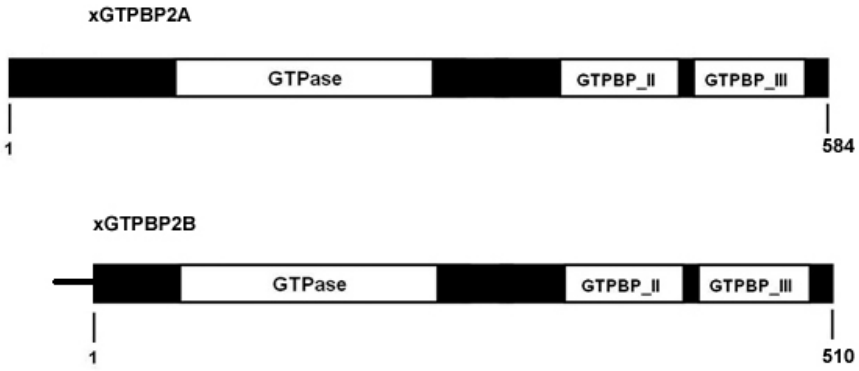


Figure 2.1: Schematic representation of GTPBP2A and GTPBP2B. GTPBP2A and GTPBP2B have the same aminoacid sequence except GTPBP2B is missing 74 aminoacids at the N-terminus. GTPBP2A and GTPBP2B have an N-terminal GTPase domain, followed by two domains, that are related to elongation factor RNA binding domains, and conserved between GTPBP1 and GTPBP2.

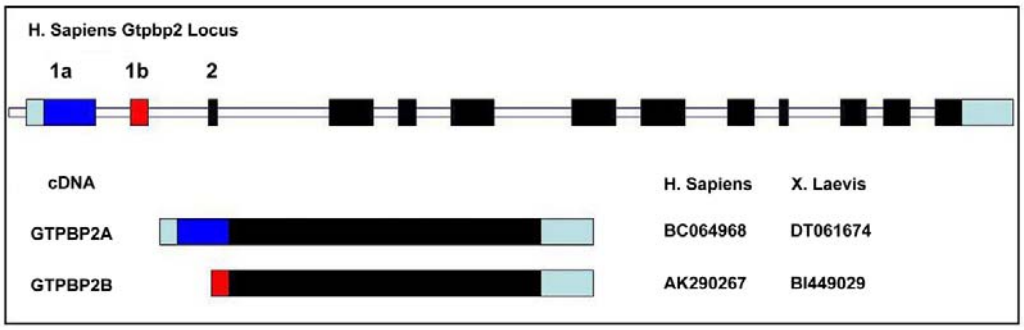


Figure 2.2: GTPBP2 human genomic locus exon-intron boundaries. Analysis using human cDNAs BC064968 and AK290267 shows that GTPBP2A and GTPBP2B are products of the same gene but start by alternative exons. Xenopus cDNAs corresponding to their human counterparts are listed.

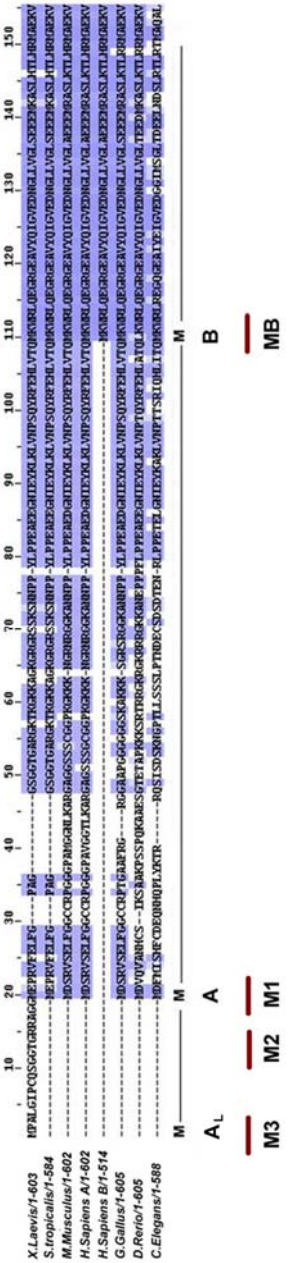


Figure 2.3: Sequence alignment of GTPBP2 from different species. H.S. stands for human GTPBP2B protein. A_L, A, and B depicts possible starts sites of GTPBP2A, and GTPBP2. Morpholinos designed against GTPBP2 are shown as M1-to-3, and MB.

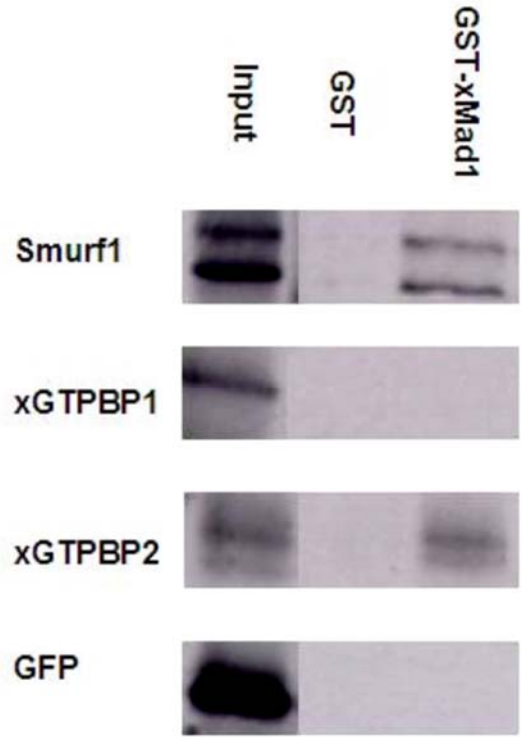


Figure 2.4: GTPBP2 interacts with Smad1 in vitro.
xGTPBP1 and xGTPBP2 were in vitro translated using rabbit reticulolysate system. GST and GST-xMad1 proteins were purified from bacteria. Purified GST tagged proteins are incubated with in vitro translated GTPBPs, and pulled down by GST beads. xGTPBP2 interacts GST-Smad1 in vitro whereas xGTPBP1 does not. Smurf1 was used as a positive control and GFP as a negative control.

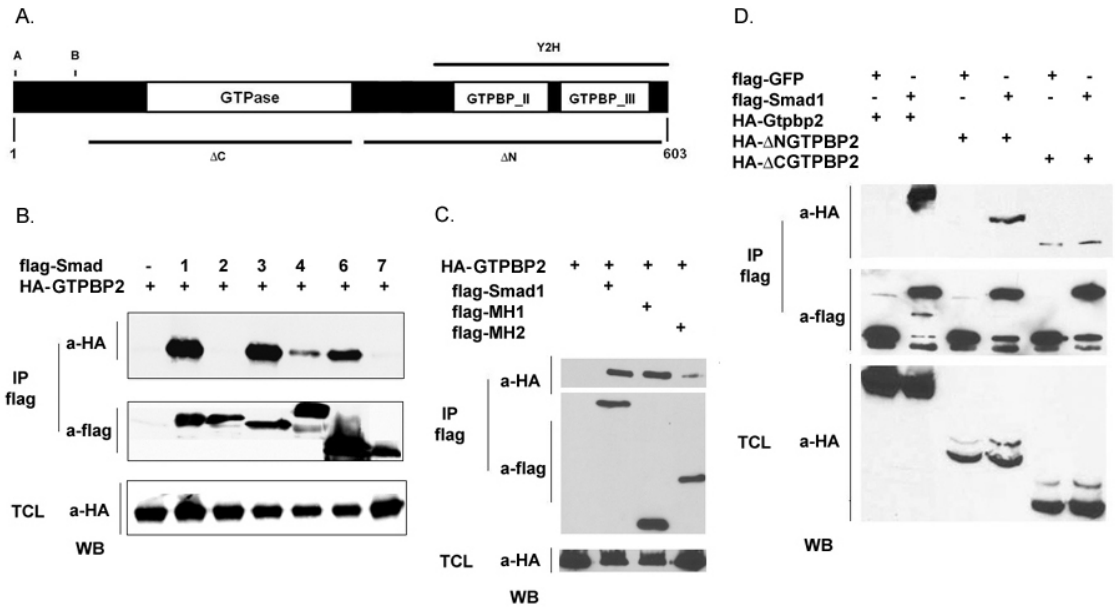


Figure 2.5: GTPBP2 interacts with Smad proteins.
A. Schematic representation of GTPBP2, and its conserved domains. Figure also shows different constructs used in interaction assays. Symbols A and B indicate possible alternative translation start sites for different GTPBP2 forms. **B.** Flag-Smad constructs were-co-expressed with HA-GTPBP2B in HEK293T cells. Cell lysated were co-immunoprecipitated using Flag-agarose beads, and analyzed by Western Blotting using α-HA-HRP, and α-F M2 antibodies. Lower panel shows total cell lysate GTPBP2B levels, top two panels show immuno-precipitated proteins. **C.** Flag-xSmad1, flag-MH1 domain, and flag-MH2 domain constructs were co-expressed with HA-GTPBP2B, and analyzed the same way as in B. **D.** HA-tagged GTPBP2B, ΔN GTPBP2B, and ΔC GTPBP2B constructs of GTPBP2B co-expressed with flag-xSmad1 in COS cells, co-immunoprecipitated, and analyzed as in B.

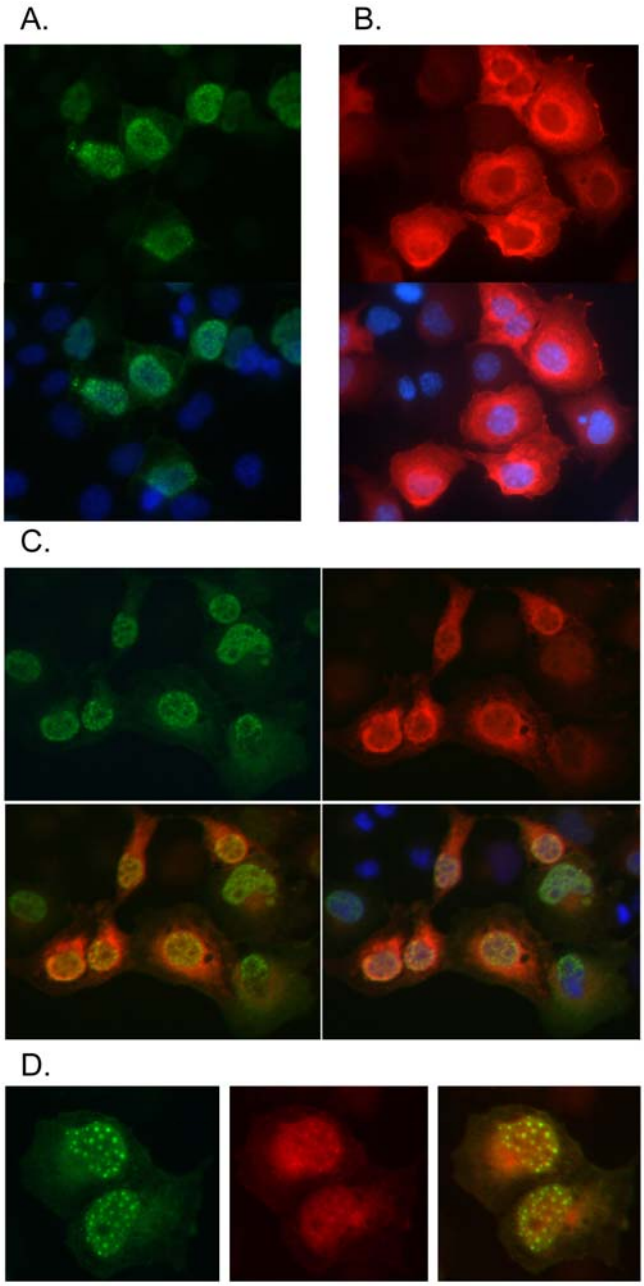


Figure 2.6: GTPBP2B is nuclear protein, and co-localizes with Smad1 in COS1 cells. COS1 cells were transfected with HA-xGTPBP2B (green) and myc-xSmad1 (red). **A.** GTPBP2B is localized primarily in the nucleus, counterstained with DAPI. **B**.Smad1 is mostly in the cytoplasm but localizes to nucleus in some cells. **C.** When co-transfected GTPBP2B and Smad1 co-localize in the nucleus in a speckled pattern. **D.** shows localization of Smad1 and GTPBP2 to nuclear foci.

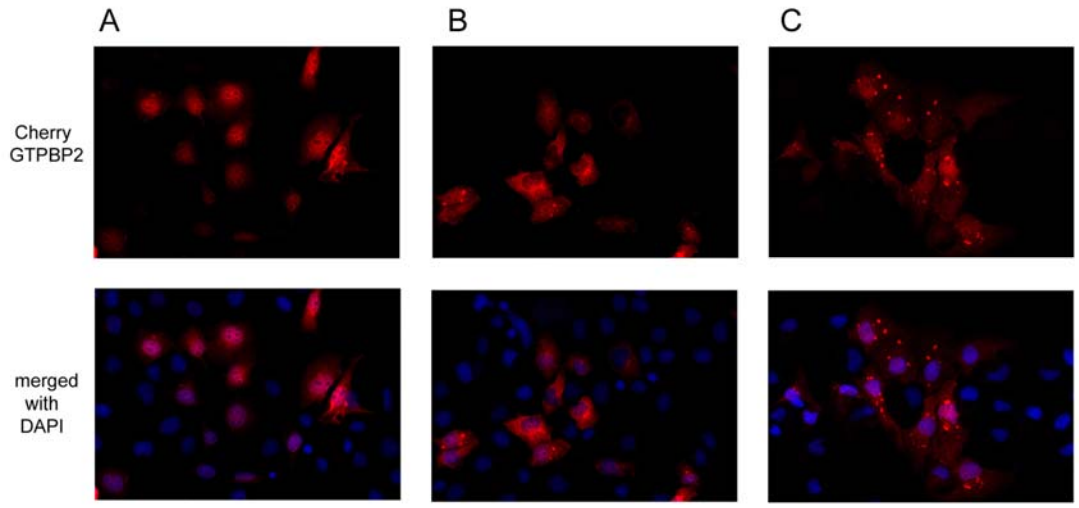


Figure 2.7: shows localization of GTPBP2A, and GTPBP2B in COS cells. Cherry-tagged-GTPBP2 clones were expressed in COS cells. **A.**GTPBP2B is a nuclear protein. **B.** GTPBP2A has a more cytoplasmic localization but at high levels of expression, GTPBP2A localizes to both cytoplasm and nucleus **(C)**.

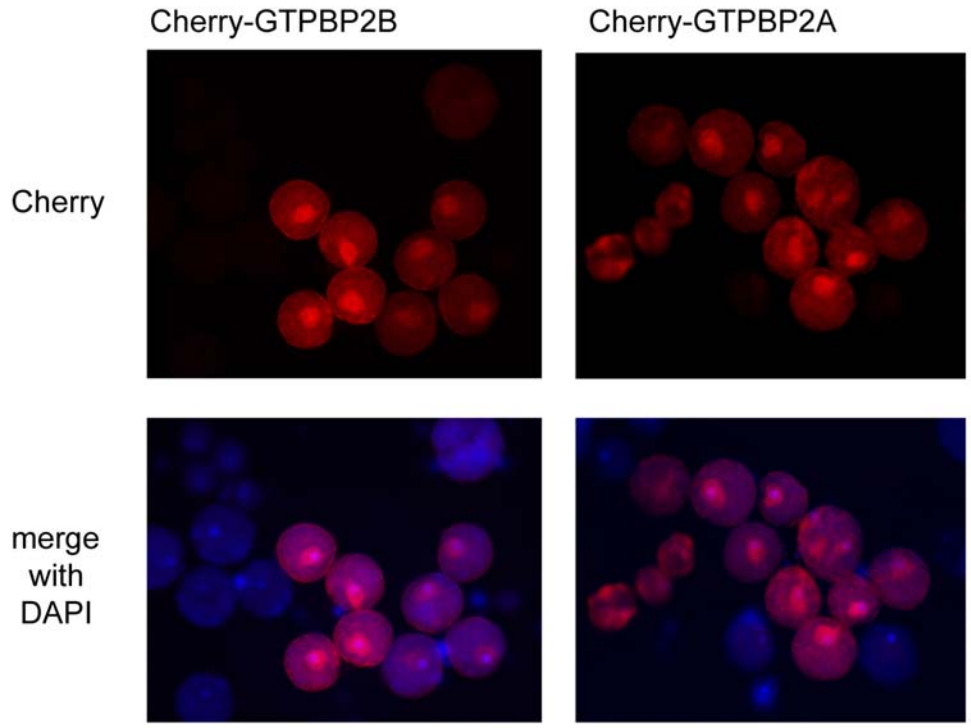


Figure 2.8: GTPBP2A and GTPBP2B are nuclear proteins in dissociated Xenopus animal cap cells

Chapter 3: GTPBP2 is a positive regulator of TGF-β signaling pathways, and is required for embryonic patterning in Xenopus.

3.1 Introduction

In embryonic development, TGF-β family ligands play essential roles in induction and patterning of all primary embryonic tissue layers, endoderm, mesoderm, and ectoderm, and they continue to be expressed in dynamic patterns at later stages of development to modulate organogenesis and growth. TGF-β /activin/nodal type ligands regulate mesoderm and endoderm induction, gastrulation movements, and left-right asymmetry, whereas BMP type ligands are involved in the induction of epidermal fates and regulation of dorsal ventral patterning of all tissue layers by imposing a ventral fate on both mesendodermal and ectodermal tissue during gastrulation and neurulation (Thomsen, 1996; Harland and Gerhardt 1997; Heasman, 2006; Wardle, 2006; also see Chapter I).

In Chapter II, I showed that GTPBP2 interacts with Smad1, Smad3, Smad4, and Smad6. Since GTPBP2 binding to Smads is not pathway specific and it can also bind to co-smad, Smad4 and inhibitory Smad, Smad6, it is hard to predict the outcome of overexpression experiments. I have employed gain-of-function and loss-function approaches to understand the nature of this interaction between Smads and GTPBP2. I found that overexpression of GTPBP2 induces mesoderm in animal cap explants, enhances Activin/Nodal and BMP signaling, and was able to rescue inhibition of BMP

signaling by Smad6. Therefore, I concluded that GTPBP2 is a positive regulator of TGF-β signaling.

The primary approach to knockdown genes in Xenopus is the use of antisense morpholinos, which can be designed to block mRNA translation or pre-mRNA splicing (Eisen and Smith, 2008; www.genetools.com). I designed morpholinos against potential initiation codons of GTPBP2. Knock-down of GTPBP2 revealed that it is necessary for BMP signaling, and mesodermal patterning.

3.2 Results

3.2.1 GTPBP2 overexpression in embryos induces secondary axis

I began investigating GTPBP2 function by testing whether it had any effects on Xenopus embryos when ectopically expressed. Synthetic mRNA coding for GTPBP2A and GTPBP2B variants were injected into embryos at 2-4-8 cell stages targeting different germ layers. Over-expression of GTPBP2B in embryos caused to a variety of defects in embryos depending on the timing of the injection and the region they were injected to. On the other hand, GTPBP2A did not perturb embryonic development. When GTPBP2 was injected into embryos prior to the first cell division, it caused gastrulation to stop; a phenomenon observed, when nodals/smad2 are overexpressed (Figure 3.1A). GTPBP2B injection into dorsal marginal zone, future organizer tissue, resulted in disruption of anterior tissues, with embryos showing mild ventralization. At higher levels, GTPBP2B injection into DMZ also resulted in gastrulation defects (Figure 3.1B). When GTPBP2B was injected into the ventral marginal zone (VMZ) of embryos at the 4-cell stage, which targeted the prospective ventral and posterior mesoderm, Injection of GTPBP2B

triggered formation of a secondary dorsal axis, similar to the sort obtained with a low dose of nodal or activin ligand (Thomsen et al., 1990), Smad2 (Baker, and Harland, 1996), or a BMP inhibitor (e.g. noggin or chordin, (Sasai et al., 1994)) (Figure 3.2.A). I further characterized these altered tissues by explanting injected VMZ tissue at early gastrulation and analyzing the expression of several early mesoderm markers. The VMZ explants from these embryos ectopically expressed organizer markers, Chordin and Goosecoid, along with ventral markers Vent1, Vent2 and Wnt8 (Figure 3.2B), which probably reflects unaffected endogenous ventral tissue, or perhaps concomitant ventral marker gene induction by GTPBP2.

3.2.2 GTPBP2 induces mesoderm in animal cap explants

Ectopic expression of GTPBP2B in the VMZ yielded phenotypes indicative of effects on mesoderm development. To determine more directly whether GTPBP2 affects mesoderm induction or patterning, I turned to the Xenopus blastula animal cap assay and tested each GTPBP2 variant for its ability to induce mesoderm. Overexpression of GTPBP2A and GTPBP2B in animal caps induced mesendoderm in a dose-dependent manner, as indicated by activation of a variety of BMP and nodal/activin target genes and region-specific germ layer markers (Figure 3.3 and 3.4A). These included general mesendodermal genes Mixer and Mix.2, pan-mesodermal genes eomesodermin and brachyury, ventral mesodermal genes Xhox3, wnt8 and vent1, and organizer/dorsal mesendodermal genes goosecoid, chordin, lim1, cerberus and siamois. GTPBP2A and GTPBP2B induced a qualitatively similar set of marker genes, but GTPBP2B was distinctly more potent, inducing a higher level of marker gene expression than GTPBP2A, when equal amounts of mRNA were injected. These results were consistent with the

ability of GTPBP2B, but not GTPBP2A to induce an ectopic axis in embryos. GTPBP2B is clearly the most potent form of GTPBP2, but each form of GTPBP2 was sufficient to trigger a mesendodermal program when overexpressed in Xenopus embryos. To address this issue, I compared the relative amounts of each GTPBP2 protein produced in the Xenopus embryo for a given dose of mRNA. I found that GTPBP2B protein levels were 5-fold higher than GTPBP2A (Figure 3.4B). To normalize mRNA translation efficiency, each GTPBP2 cDNA was cloned so that the coding regions had identical flanking, HA-tag and adjacent Kozak sequences. I eliminated variation in mRNA quality as a reason for the observed differences by repeating these assays multiple times with independent sets of GTPBP2 mRNAs synthesized side by side. Thus, when protein levels are normalized both forms of GTPBP2 had similar mesendoderm inducing activity for most of the genes analyzed.

3.2.3 GTPBP2 enhances BMP and Nodal signaling in animal cap explants.

GTPBP2 interacts with Smad proteins and can induce mesoderm when overexpressed in Xenopus animal caps. I therefore set out to test whether GTPBP2 would influence BMP or nodal/activin signaling pathways, with the prediction that the effect would be a positive one. I first tested its effects on BMP or nodal direct response genes in Xenopus animal caps. The caps were injected with a limiting dose of BMP-4 or nodal ligand (Xnr2), and increasing amounts of GTPBP2. Overexpression of GTPBP2A together with ligands in the caps synergistically enhanced expression of BMP responsive genes Vent1, and Xhox3, and Activin/Nodal responsive genes Eomes, and Mixer (Figure 3.5A, B). The interaction of GTPBP2 with R-Smads strongly suggests that GTPBP2 acts

on the Smads in some manner to boost BMP and nodal signal transduction. I tested whether GTPBP2 cooperates with Smads in mesoderm induction and direct response gene activation. Consistent with expectations, co-expression of GTPBP2 with either BMP-specific Smad1 or nodal/activin-specific Smad2 enhanced the ability of these Smads to induce mesoderm and activate direct response genes in animal caps (Figure 3.6).

To further substantiate whether GTPBP2 acts directly on BMP (Smad1/5/8) or nodal/activin (Smad2/3) pathways, and to assess the generality of the effects I observed in Xenopus embryos, I tested whether GTPBP2 would affect BMP or TGF-β driven reporter gene activation in the TGF-β and BMP responsive human HepG2 hepatoma cell line. These cells were co-transfected with GTPBP2 and either BMP or TGF-β reporter constructs, consisting of synthetic Smad1 (12x-BRE) or Smad2/3 (9xTRE) binding elements. Treatment of these cells with the correspondingly appropriate growth factor demonstrated that GTPBP2 enhanced the activation of these reporter genes (Figure 3.5C, D). Also, GTPBP2 overexpression alone was capable of activating the BMP reporter, but not the TGF-β reporter. I concluded that GTPBP2 is directly involved in Smad signal transduction, where it exerts a positive input on both the Activin/Nodal/ TGF-β and BMP branches of the TGF-β superfamily.

3.2.4. GTPBP2 rescues BMP inhibition by Smad6

Besides its interaction with R-Smads, GTPBP2 interacts significantly with Smad6, an inhibitory Smad that primarily blocks the BMP pathway by inhibiting Smad1/5/8 signal transduction. Consequently, when Smad6 is overexpressed in animal caps (an ectodermal tissue explant), they differentiate into neural tissue (Vonicka and Brivanlou 2006). I wanted to know how GTPBP2 would affect Smad6 action, and in

particular whether or not it would enhance the inhibitory effects of Smad6 (in view of its positive effect on R-Smads). Figure 3.5E shows that injection of Smad6 mRNA alone induced neurectodermal markers, NCAM and XAG, reflecting inhibition of endogenous BMP signaling. Injection of GTPBP2 alone induced mesodermal markers Xbra and Xpo (a posterior mesodermal marker), as observed above. However, when a low dose of GTPBP2 was introduced along with Smad6, the neuralizing effects of Smad6 were abolished. Smad6 combined with the high dose of GTPBP2, quenched neural induction as well, but also triggered a more robust and dorsolateral mesoderm response compared to GTPBP2 alone. These results showed that GTPBP2 does not enhance Smad6 activity, but instead it appears to block Smad6, and rescue BMP signaling inhibition. In the presence of Smad6, GTPBP2 also induced mesodermal genes of dorsal character; muscle-actin and MyoD.

3.2.5 Gtpbp2 shows a dynamic expression pattern during early Xenopus development.

To understand the physiological significance of interaction between Smads and GTPBP2, I first carried out in situ hybridization and RT-PCR analysis to determine where and when Gtpbp2 is expressed in developing Xenopus embryo. In situ hybridization analysis shows that Gtpbp2 transcripts are maternally present, and localized to the animal pole in early cleavage and blastula stages (Figure 3.7A, B). During gastrulation, Gtpbp2 signal is very diffuse, and not localized to specific tissues, if at all present (data not shown). At neurula stages, Gtpbp2 is expressed in anterior neural tissue, and later it is localized to developing nervous system and somites. (Figure 3.7C, D) In early tadpole stages, Gtpbp2 is distinctly localized to developing somites (Figure 4E, F),

and at stage35 embryos, it is also localized to ventral blood islands, and diffusely to different head structures in addition to the somites (Figure 4G). The embryonic expression pattern of Gtpbp2 is similar to the previous reports showing that in adult mouse tissues, it is expressed in brain, skeletal muscle and blood cells (Kudo et al., 2000). Since Gtpbp2A and Gtpbp2B mRNAs have only a few nucleotide differences, in situ probe is made using Gtpbp2A cDNA. So the expression profile in these experiments is not specific to the different proteins made from GTPBP2 gene.

I then assayed Gtpbp2 transcript levels at different embryonic stages by quantitative RT-PCR. RT-PCR experiments confirmed that Gtpbp2 transcripts are deposited maternally, and stay at relatively stable levels during blastula stages. The mRNA levels start decreasing at the onset of gastrulation and stay low until the end of gastrulation stages (Figure 3.8). Zygotic expression of gtpbp2 starts at stage 14, and increases at following time points. From these experiments, I concluded that Gtpbp2 mRNA is maternal, and it is dynamically down-regulated during gastrulation, which makes Gtpbp2 a good target for loss-of-function experiments using translation interfering morpholinos in early development.

3.2.6 GTPBP2 is essential for axial patterning in early Xenopus development.

Nodal/activin and/or BMP signals are required for induction and patterning of the three primary germ layers of Xenopus embryos, and all vertebrate embryos (De Robertis and Kuroda, 2004; Schier and Talbot, 2005). GTPBP2 is maternally expressed in the egg and throughout the critical early induction and pattering phases of the Xenopus germ layers. Does GTPBP2 exert an essential function in Xenopus development and does it

affect the relevant TGF-β pathways? To address these key questions I performed protein knock-down experiments by utilizing translation-blocking antisense morpholino oligos (MOs). There existed some ambiguity in defining the N-terminus of GTPBP2A in Xenopus, due to two in-frame Met codons within the first 20 aa's of the longest ORF. The first of these Met codons is only conserved in Xenopus, and lacks a Kozak consensus sequence suggesting that it is less likely to be translated , if at all. I term this putative form GTPBP2A_L (Figure 2.3). I have taken this fact into consideration when designing morpholinos for loss-of-function experiments. Morpholinos designed against GTPBP2 are shown as M1-to-4 (Figure 2.3 and 3.9).

The start Met sites of the potential A, A_L, and B versions of GTPBP2 were targeted with translation-blocking MOs, which I injected into either all embryonic cells or specifically-fated blastomeres. Morpholino M3 targets the most upstream Met codon that defines the putative start site of GTPBP2A_L. Injection of at up to 100ng of M3 did not cause any embryonic defects, regardless of where it was targeted in embryos (Figure 3.9). Morpholinos targeting the Gtpbp2A or Gtpbp2B, however, caused significant developmental defects. Morpholinos M1 and M2, which target GTPBP2A, and inhibit in vitro translation (Figure 3.10A), caused severe patterning defects (Figure 3.10B, 3.11) that included head loss and axial truncation. The M1 morpholino, which has a sequence that overlaps the start codon, was more potent than the M2 morpholino that binds to a sequence more 5' to the start site (Figure 2.3 and 3.9A). However, both caused essentially similar phenotypes. Morpholino MB, which targets the start site of putative Gtpbp2B form (Figure 3.9B, C), caused a milder phenotype that became apparent at tadpole stages,

leading to shortened body axis and defective somites. I concluded that both GTPBPA and GTPBP2B are physiologically significant for normal development.

I characterized the knockdown phenotypes in more detail, focusing on the effects of the GTPBP2A-specific MOs, M1 and M2, because they produced very early (gastrulation) phenotypes that suggested a potential function in endogenous TGF-β signaling. Before I characterized the GTPBP2A morphant embryos, I verified that the M1 and M2 morpholinos blocked translation of a C-terminal myc-tagged Gtpbp2 mRNA injected into embryos. A random sequence (non-targeting) control MO and a 5-mismatch control version of M2 did not inhibit target protein translation, nor was translation of a co-injected GFP mRNA blocked by any of the morpholinos (Figure 3.10A). When injected laterally into both blastomeres at the two cell stage, or into the two dorsal/anterior cells of four cell embryos, both of the GTPBP2A MOs caused severe axial defects, with overt changes appearing at the onset of neurulation as a lack of neural plate folding and dorsal fusion (Figure 3.9). The embryos looked as though development became stalled at stage 14, yet they survived for days afterwards (Figure 3.11). At the most effective MO doses, The GTPBP2A morphants lacked a head, axial structures, somites and tail when scored at swimming tadpole (stage 35) (Figures 3.10B. and 3.11).The severe morphant phenotypes generated by dorsal injection of M1 or M2 could be rescued by co-injection of Gtpbp2 mRNA along with either MO. Interestingly, GTPBP2 morphants were rescued most convincingly only when I injected a cocktail of Gtpbp2A and Gtpbp2B mRNA (Figure 3.10B). I also tested whether knockdown of GTPBP2A has an effect on posterior-ventral tissues, independent of effects on dorsal tissues. Indeed the M1 and M2 mopholinos disrupt tail and posterior development

without effecting dorsal tissues when injected into the ventral two blastomeres at the four-cell stage (Figure 3.11). The results of these targeted injections indicate that GTPBP2 is required for induction and/or patterning of dorsal and ventral tissues, and implicate the mesoderm as a sensitive target.

To determine more precisely whether and what kind of defects occur in GTPBP2 morphants, I scored a range of mesendodermal molecular markers by whole mount in situ hybridization on embryos at gastrulation. Fig. 5C-F demonstrates that expression of a variety of markers was significantly impaired or eliminated in GTPBP2A morphants. The posterior-ventral markers, xpo, wnt8, and myoD were most affected (Figure 3.10C), displaying complete loss of expression in marginal zone cells that received the MO. The expression of Organizer markers, chordin, goosecoid and frzb1, was also reduced significantly and reproducibly, yet not eliminated, in dorsally-targeted morphants (Figure 3.10D). On the other hand, mesendodermal markers, mix.2 and mixer, and endoderm-specific marker sox17a were not affected by MO treatments (Figure 3.10D). Furthermore, expression of two general mesodermal markers, the T-box genes brachyury and antipodean (the zygotic form of VegT/Brat) were affected unequally. Brachyury expression was nearly eliminated while Apod was unaffected in morphants (Figure 3.10F). I concluded that GTPBP2 is essential for normal mesodermal patterning, but that some mesendodermal target genes display a differential sensitivity that might reflect GTPBP2 pathway specificity or bias in the embryo.

3.2.7. GTPBP2 is required for normal BMP and nodal signaling

I have shown that GTPBP2 can boost BMP or nodal/activin/ TGF-β signaling in embryos or cultured mammalian cells, and that GTPBP2 is necessary for normal

embryonic development. The severity of the phenotypes and the differential effects of GTPBP2 knockdown on early marker genes also suggest that more than one TGF-β signaling pathway might be affected in morphant embryos. The fact that GTPBP2 can interact with Smad4 (and in addition to that, Smad1, Smad3) makes it likely that both of the major Smad branches of TGF-β signaling could be affected by GTPBP2. To investigate if GTPBP2 is a common factor necessary for both BMP and Nodal signaling in vivo, I analyzed the effects of GTPBP2 knock-down on BMP4 and Xnr2 induced gene expression in animal cap explants. Figure 3.12 demonstrates that knockdown of GTPBP2A with morpholinos M1 or M2 inhibited BMP4-mediated gene induction in animal caps. The levels of BMP-inducible, ventral mesoderm specific genes, Vent1, Wnt8 and Xhox3 were reduced up to 80% when Gtpbp2 was knocked-down. As observed previously in whole embryos, the M1 morpholino was a more potent inhibitor of BMP-mediated gene induction than M2 (Figure 3.12A). Importantly, MO inhibition of BMP induced gene expression was rescued by co-injection of limiting amounts of Gtpbp2 mRNA, which alone did not induce significant marker gene expression (Figure 3.12C).

To examine more directly the effects of GTPBP2 loss of function on BMP gene responses, I monitored endogenous BMP/Smad1 signaling in animal caps using a luciferase reporter gene driven by the Vent2-promoter, a direct target gene of BMP4. Animal cap tissue has a low but significant level of endogenous BMP signaling, which drives the BMP target genes Vent1 and Vent2 when caps are cultured without perturbation. Animal caps injected with the Vent2-luciferase promoter and a control MO had a significant level of reporter activity, but injection of the GTPBP2 M1 morpholino

reduced this activity by about 50% compared to the control samples (Figure 3.12B). Thus, I concluded GTPBP2 is required for endogenous BMP signaling.

To assess whether endogenous GTPBP2 functions in Activin/Nodal signaling in animal caps, I performed a set of experiments similar to those above, treating animal caps with nodal ligand in the presence of GTPBP2 or control MOs. Animal caps were injected with a limiting dose of Xnr2 plus control or GTPBP2 morpholinos. Contrary to my observations with BMP treatment, I found that when GTPBP2 levels were knocked down, the expression of Xnr2 responsive genes Mixer, Mix2, and Gsc increased substantially (Figure 3.13A). To assess whether this potentiated nodal response to GTPBP2 knockdown in animal caps happens in whole embryos, I employed a TGF-β /nodal luciferase reporter gene driven by a 9x-CAGA, Smad3 response element (Dennler et al., 1998). As observed with caps, I found that knockdown of GTPBP2 in the embryo increased the reporter activity in a MO dose dependent manner (Figure 3.13B). Therefore, I concluded that GTPBP2 is not necessary for Activin/Nodal signaling, and it may act in an inhibitory way indirectly by activating BMP signaling.

3.2.8 GTPBP2 is not required for Smad1 localization and phosphorylation

The cellular localization of GTPBP2 suggests it has a nuclear function in Smad signaling. To investigate the possibility that GTPBP2 is required for Smad1 nuclear localization, I analyzed the location of GFP-Smad1 in dissociated Xenopus animal cap cells treated with either control or GTPBP2 MOs. I observed that Smad1 was localized to the nucleus of control MO treated cells (Figure 3.14A), consistent with previous findings that such cells maintain nuclear Smad1 due low level active BMP signaling (Simeoni,

and Gurdon, 2007). However, GTPBP2 knock-down did not cause any change in this Smad1 localization pattern. I then tested a more general question of whether GTPBP2 knock-down affects phosphorylated Smad1/5/8 levels. I found no change in phopho- or total Smad1/5/8 protein levels in Gtpbp2 morpholino treated animal cap explants (Figure 3.14B). I also examined whole embryos treated with a GTPBP2 MO and found no change in the level of P-Smad1/5/8. Hence, I concluded that GTPBP2 knock-down does not affect the stability, localization or phosphorylation status of Smad1.

3.3 Discussion

TGF-β signaling pathways control several fundamental aspects of development including axis formation, body patterning, and morphogenesis. At the cellular level, TGF-β regulates cell proliferation, differentiation, migration, and apoptosis. Because of its key role in these processes, a plethora of regulators are evolved to modulate TGF-β signaling. In this chapter, I showed that GTPBP2, a large GTPase, is a positive regulator of TGF-β signaling. GTPBP2 interacts with Smads, induces mesoderm, and enhances BMP and Nodal/TGF-β induced gene expression. GTPBP2 is required for embryonic patterning, and BMP signaling in Xenopus development.

I suggest that GTPBP2 functions as a general factor for Smad signaling in both major branches of the TGFβ superfamily, BMP and Activin/Nodal. This proposition is supported most directly by the fact that GTPBP2 interacts with BMP-specific Smad1, Activin/Nodal specific Smad3, and the common R-Smad signaling partner, Smad4. GTPBP2 also binds to Smad6, an inhibitory Smad that blocks BMP signaling by interfering with Smad1/5 – Smad4 complex formation. Gain and loss of function

experiments demonstrate that GTPBP2 governs responses to BMP, Nodal and TGFβ ligands in embryos and cultured cells. In Xenopus animal cap explants, GTPBP2 overexpression enhances mesoderm induction and activation of BMP or Nodal direct response genes by the corresponding ligands. GTPBP2 also enhances Smad1 and Smad3 reporter gene activation in human HepG2 cells, demonstrating that the effect of GTPBP2 on TGFβ signals is likely a general property of vertebrate cells. Additional findings that GTPBP2 overexpression alone induces mesoderm in Xenopus animal caps, and that GTPBP2 also synergizes with Smad1 and Smad2 to induce mesodermal genes, provide support that GTPBP2 engages TGFβ pathways at the level of the signaling Smads.

GTPBP2 appears to exert its actions on Smad signaling in the nucleus, where it is predominantly and constitutively localized. GTPBP2 is capable of recruiting a fraction of Smad1 to the nucleus, even in the absence of BMP signals, where the two proteins co-localize to distinct nuclear speckles or foci, and a low level diffuse pattern throughout the nucleus. In mammalian cells, Smad1 is mostly cytoplasmic at steady state, under non-signaling conditions, but Smad1/5 has been previously found to localize in subnuclear foci (Janknecht et al., 1998; Yoshida et al., 2000) that resemble the GTPBP/Smad1 speckle pattern I observed. In particular, activated Smads 1 and 5 were found in subnuclear speckles in association with Runx proteins at sites of active transcription (Zaidi et al., 2002). Nuclear speckles have been variously described as locations of DNA replication, gene transcription, splicing factor assembly, pre-mRNA processing and intranuclear transport (Zimber et al., 2004). Although I have not determined the molecular nature of GTPBP2-containing speckles, they are consistent with the possibility that GTPBP2 functions in transcription and/or post-transcriptional processing of Smad

target genes. In addition, knockdown of GTPBP2 in Xenopus animal cap cells did not change the phosphorylation status of Smad1, which argues against the possibility that GTPBP2 functions at the receptor level, such as operating as a receptor adaptor or somehow controlling access of Smad1 to Type I receptors. Furthermore, the fact that GTPBP2 rescues Smad6-dependent neuralization of animal caps argues that GTPBP2 acts in the BMP pathway at the level of Smad1/5/8-smad4 complexes or further downstream, to promote signaling. Hence, I concluded that GTPBP2 functions as a nuclear co-factor in TGFβ/Smad signaling.

GTPBP2B is a more active form of GTPBP2. Human Gtpbp2 gene codes for two forms of GTPBP2 protein, GTPBP2A, and GTPBP2B through alternative use of starting exons. xGTPBP2B, which is missing the first 74aa's, acts as a more potent mesoderm inducer than GTPBP2A. When overexpressed in embryos GTPBP2B induces a partial secondary axis and gastrulation defects. Although most of the functional discrepancy can be explained by different over-expression levels, I was not able to express GTPBP2A form at a level which will give same activity as high dose of GTPBP2B in animal cap explants. Furthermore, GTPBP2A does not induce secondary axis, or block gastrulation in embryos. Therefore, GTPBP2A, and GTPBP2B may have different in vivo activities. Morpholino experiments targeting GTPBP2B form shows that it is physiologically relevant. GTPBP2B morpholino led to late stage axial defects primarily in the somites.

The discrepancy between the potencies of GTPBP2A and GTPBP2B may be due the difference in GTPase activity. I did not directly test this but I gathered clues from overexpression assays using potential dominant negative and constitutively active mutants of GTPBP2. I also aimed to use these dominant negative mutants to interfere

with endogenous function of GTPBP2. GTPBP2 has a GTPase domain with four signature motifs that are essential for GTP binding and hydrolysis (Vetter and Wittinghofer, 2001) (Figure 3.15). Mutations involving GTPase signature motifs are isolated and well described for Ras and eEF1A proteins (Lowy and Willumsen, 1993; Carr-Schmid, 1999). I introduced these mutations and test their effects in embryos (Figure 3.15). GTPBP2B forms with dominant negative mutations retained their activity to induce mesoderm in animal caps, and secondary axis in embryos. Similarly, I did not observe an enhanced activity in GTPBP2B forms with mutations that potentially activate it. Similarly, introducing these mutations to GTPBP2A form did not cause any phenotype in embryos. Therefore, the rate of GTPase activity may not be important for GTPBP2 function.

GTPBP2 is required for BMP signaling during Xenopus embryonic development. GTPPBP2 transcripts are maternally present and localize to animal half of the embryo during blastula stages. GTPBP2 morphants show extensive axial and patterning defects, including loss of head, somites and tail, indicating an early role in germ layer specification for GTPBP2. Loss of GTPBP2 in animal caps results in drastic reduction of BMP responsive genes and reporter activity but did not inhibit Xnr2 responsive gene expression. On the contrary, GTPBP2 knock-down increased the response to Xnr2 in animal cap explants and activity from a TGF-β reporter construct in embryos. Consistent with the explant and reporter assays, in gastrula stage embryos, I did not see an effect on Nodal target genes Mix2, Mixer, and eomesodermin but BMP responsive ventral mesoderm genes Wnt8, Xpo, and MyoD (Marom et al. 1999, 2005) were absent or severely downregulated in GTPBP2 morphants. I concluded that, in early development,

GTPBP2 is primarily required for BMP branch of TGF-β signaling, and dorsal ventral patterning of mesoderm. Interestingly, the levels of organizer genes Chordin, and Goosecoid, and pan-mesodermal gene Xbra were reduced as well. All of these genes are under complex transcriptional regulation. Goosecoid promoter has Activin/Nodal and Wnt responsive elements, (Watabe et al., 1995) and Chordin is induced indirectly by Nodal signaling through Goosecoid, and by Wnt signaling (Sasai et al., 1994). Both Chordin and Goosecoid are induced by Nodal in a dose dependent manner. (Piccolo et al., 1999) Similarly, Brachyury is induced by FGF, low levels of Nodal, and Wnt signaling, (Vonica and Gumbiner, 2002) and high levels of Activin/Nodal signaling blocks Brachyury expression (Latinkic et al., 1997). Although it is possible to interpret decreased Brachyury levels as a result of higher Activin/Nodal activity, I believe effects of GTPBP2 knock-down on organizer genes, and Brachyury indicate that GTPBP2 may be involved in other signaling pathways that are critical for organizer formation and mesodermal pattterning. Alternatively, GTPBP2 might be required for this subset of Activin/Nodal signaling target genes.

GTPBP2 is highly similar to GTPases that are involved in translation machinery; eEF1A, eRF3 (GSPT1-2), and HBS1. The canonical function of eEF1A is to facilitate peptide chain elongation during mRNA translation. However, eEF1A also has a wide array of other functions, including activation of signaling enzymes PLCγ1 and SK1 (Chang et al., 2002; Leclercq et al., 2008), regulation of actin cytoskeleton (Gross and Kinzy, 2005), and induction of apoptosis by acting as a transcriptional repressor (Rho et al., 2006). Paralogs of eEF1A; eRF3 (GSPT1-2) and HBS1 have functionally diverged and do not bind aa-tRNA (Inagaki et al., 2003). In addition to its role in translation

termination, eRF3 is involved in mRNA deadenylation and degradation (Funakoshi et al., 2007). Smad transcription factors have been shown to interact with many proteins that are functionally or structurally related to mRNA stability (Tob), splicing (Sf3b2), or has RNA binding domains (MAN1) (Osada et al., 2003; Warner et al., 2003; Yoshida et al., 2000). Among these unusual partners, Tob is well characterized. Tob and its homologs are involved in mRNA stability by regulating mRNA deadenylation (Miyasaka et al., 2008; Ezzedine et al., 2007). Studies in mice and zebrafish showed that Tob is required for dorsal ventral patterning and it inhibits both Nodal and BMP signaling by interacting with Smad1, 3, 4, 6 and 7 (Xiong et al., 2006; Yoshida et al., 2000). Interestingly, Tob and eRF3 cooperate to regulate mRNA deadenylation (Funakoshi et al., 2007). Finally, a recent paper suggests that Smads can directly bind to RNA and control DROSHA-mediated microRNA maturation (Davis et al., 2008). I believe these previous reports together with my characterization of GTPBP2 as a regulator of TGF-β signaling points out to a role for TGF-β signaling in mRNA processing.

In summary, these results demonstrate that GTPBP2 is a key component of TGF-β signaling. The interaction of GTPBP2, and biochemically related genes Tob1, Sf3b2, and xMAN1 with Smad proteins indicates that there is an unexplored link between TGF-β signaling and post-transcriptional processes such as mRNA stability and splicing through Smad transcription factors.

3.4 Materials and Methods

3.4.1 Xenopus animal cap assays and quantitative RT-PCR

Synthetic mRNAs or MOs were injected into the animal pole of 2-cell stage embryos at the following doses: 0.1-4ng GTPBP2B, 0.1-16ng GTPBP2A, 500 pg BMP4, 10 pg Xnr2, 1ng Smad1, 1ng Smad2, and 2ng Smad6 mRNA. GFP mRNA was co-injected with other mRNAs to normalize the total amount of injected mRNA. Animal caps were isolated at stage 8, cultured in 0.5x MMR, and harvested at stage 11 or 18. Ten animal caps per each treatment were pooled and total RNA was extracted as described (Alexandrova and Thomsen, 2006), followed by cDNA synthesis with Superscript II Reverse Transcriptase (Invitrogen) using oligo-dT16-20 primers (Invitrogen). Real-time quantitative PCR was performed with a LightCycler 480 System (Roche). Primer sequences and conditions were as described (Kofron et al., 2001; Yokota et al., 2003). Target gene expression levels were normalized to the expression level of ODC transcripts.

3.4.2 Reporter Gene Assays

HepG2 cells were transfected with combinations of the following plasmids using Lipofectamine 2000; inducible reporter constructs (12XBRE-Luc or 9XTRE-Luc) (0.1ug), control reporter TK-Luc (0.01ug), GTPBP2 (25-400ng). 18 hours after transfection, cells were stimulated with 300ng/ml BMP4 or 5pg TGF-β2 for another 18 hours. Cell extracts were prepared and analyzed using Dual-Glo Luciferase Assay System (Promega). Reporter Assays in Xenopus embryos were done in a similar way by

injecting 100ng 9xTRE-Luc or Vent2-Luc reporter together with 50ng SV40-Luc reporter as interrnal control to normalize the reporter activity, and 20-40ng of M1 and 5-mis morpholinos designed against GTPBP2.

3.4.3 Western Blotting

HA-tagged GTPBP2A and GTPBP2B mRNAs were injected into embryos at two cell stage at 0.1-16ng dose. Protein levels were assayed at stage 11. Embryos were lysed in PBS containing 1% Triton-X-100, and insoluble fraction was centrifuged away. Samples were run on 10% SDS gel, and detected with anti-HA-HRP antibody (Roche; 1-500).

3.4.4 In Situ Hybridization and Q-RT-PCR for GTPBP2

Whole mount in situ RNA hybridization (WISH) was performed as described (Harland, 1991). GTPBP2 mRNA levels were assayed by quantitative RT-PCR using forward primer ATGCCCGCGCTGGGCATTCC, and reverse primer TCTCTTCTCGACAACCCTA. Expression levels were normalized to the expression level of ornithine decarboxylase (ODC). Real-time quantitative PCR was performed with a LightCycler 480 System (Roche).

3.4.5 Morpholino and mRNA injections for GTPBP2 morphants

Xenopus embryos were collected and microinjected as described previously (Alexandrova and Thomsen, 2006). Morpholino Oligonucleotides (MOs) were supplied by GeneTools Inc., as follows M3: CTCCAGAGCGTACCACTTAGGAA-CC, M2:

TCCCCCTGACTGGCACGGAATGCCC, M1:CGCGGCTCCATCCCACCGG-CCCTG, 5mis: CcCGGgTCCATgCCACCcGCCgTG, MB: ATTTCATCTGGGTC-ACCAAATGCTC. Synthetic mRNAs were synthesized with mScript™ mRNA Production System (Epicentre). The effectiveness of morpholinos was assayed against a C terminally myc-tagged GTPBP2 construct containing 5' UTR. The specificity of morphant phenotype was confirmed by rescuing it with a GTPBP2 construct containing 5-nucleotide mismatches within morpholino binding region. For rescue assays, 30ng M1 morpholino was co-injected with 100pg of GTPBP2B and 1ng GTPBP2A mRNA into DMZ.

3.4.6. Smad1 phoshorylation or localization

Embryos were injected with 500pg BMP4 and 30ng M1 or control morpholinos at two cell stage,and cultured to stage 8. Animal caps were excised and cultured to stage 11. 10 caps per sample lysed and run on SDS-Page gel. Phospho-Smad1 was detected by p-Smad1 antibody (Cell Signaling, 1-1000 dilution), and Smad1 was detected by a-Smad1 antibody (Santa Cruz, 1-500 dilution). Primary antibodies were detected with IRDye 700DX goat anti-mouse, and IRDye 800CW goat anti-rabbit antibodies (Rockland Immunochemicals), images were generated using Odyssey Scanner. For Smad1 localization, 1ng GFP-Smad1 RNA was injected into two cell embryos together with 30ng M1 or control morpholino. Animal caps were excised, dissociated and cultured to stage 11 on fibronectin coated slides (Simeoni and Gurdon, 2007).

3.5 Figures

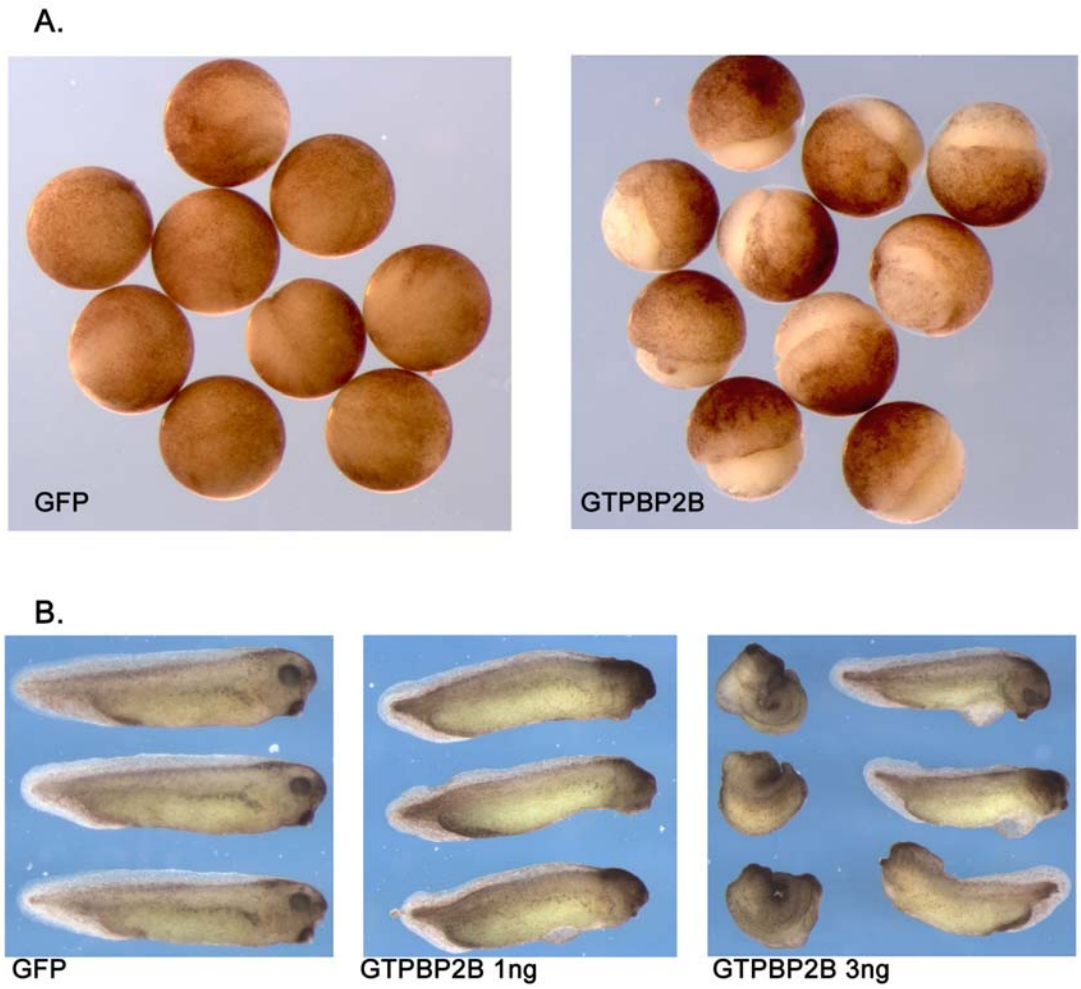


Figure 3.1: GTPBP2B blocks gastrulation and induces anterior defects. A. Injection of 2ng GTPBP2B at two cell stage into animal pole blocked gastrulation. **B.** GTPBP2B overexpression in the dorsal ventral zone resulted in dorsal-anterior defects; small head, missing eyes, and smaller cement gland. At higher levels, half of the embryos failed to gastrulate.

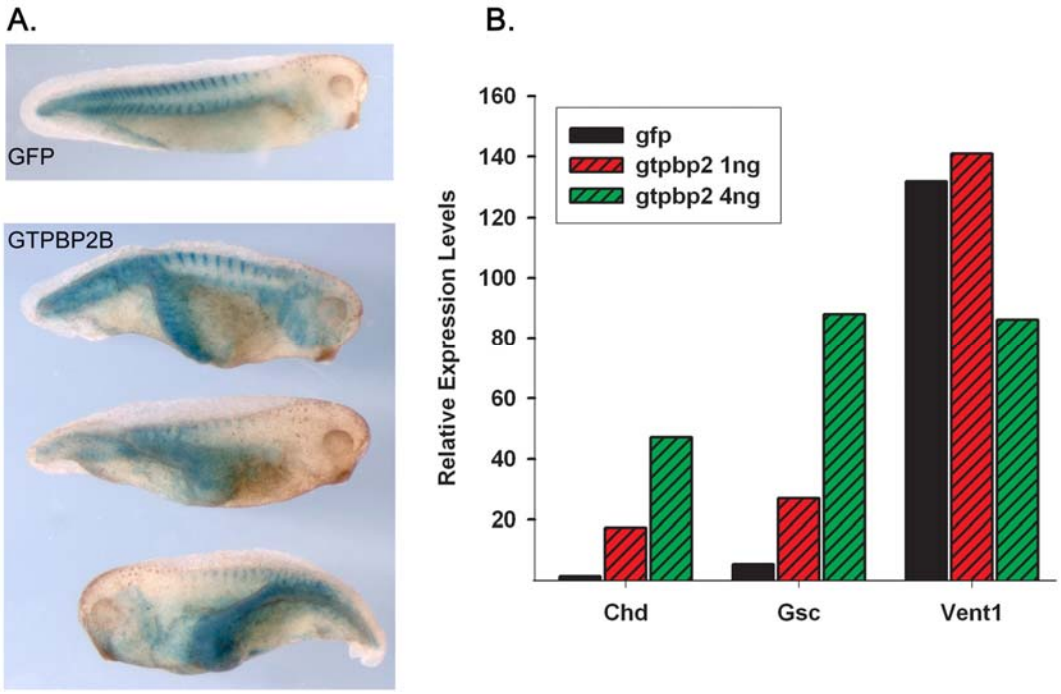


Figure 3.2: GTPBP2B induces secondary axis. A. Injection of 3ng GTPBP2B into ventral marginal zone (VMZ) induced a partial secondary axis. Blue staining is for lineage marker LacZ. **B.** Isolated ventral marginal zone tissue expressed dorsal-organizer markers goosecoid (gsc) and chordin (chd), as well as ventral marker Vent1.

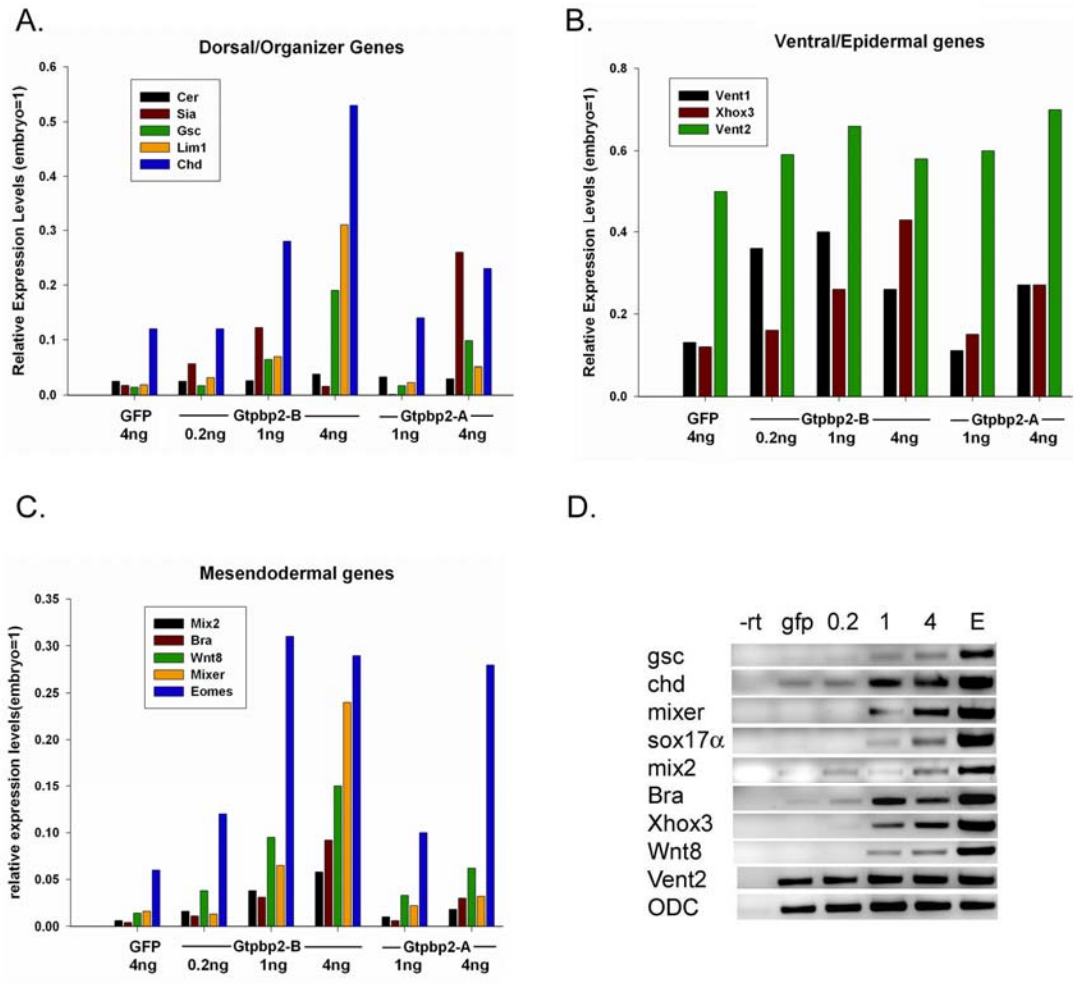


Figure 3.3 GTPBP2 induces mesodermal genes in animal cap explants. GTPBP2 induced dorsal (**A**), ventral (**B**) as well as pan-mesendodermal (**C**) markers in animal caps cultured to stage 11. Tissue markers were assayed by quantitative RT-PCR. GTPBP2B was a more potent inducer than GTPBP2A. (**D**). Similar analysis was done using semi-quantitative RT-PCR.

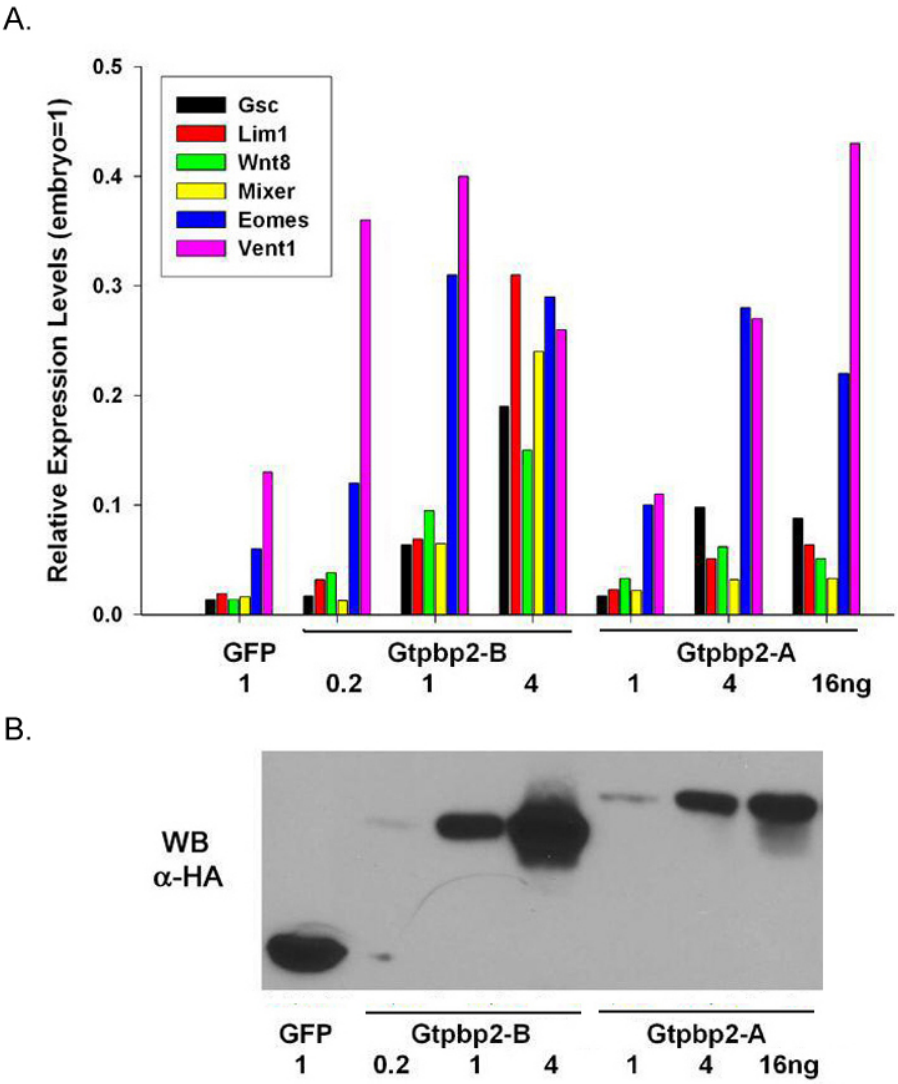


Figure 3.4: GTPBP2 induces mesendodermal genes in animal caps. B. GTPBP2 induced dorsal, ventral as well as pan-mesendodermal markers in animal caps cultured to stage 11. GTPBP2B mRNA was a more potent inducer than GTPBP2A mRNA. **C.** GTPBP2B protein accumulated to higher levels in embryos injected with Ha-tagged GTPBP2B. GTPBP2A and GTPBP2B mRNAs were injected at two-cell stage and protein levels assayed at stage 11 using anti-HA antibody.

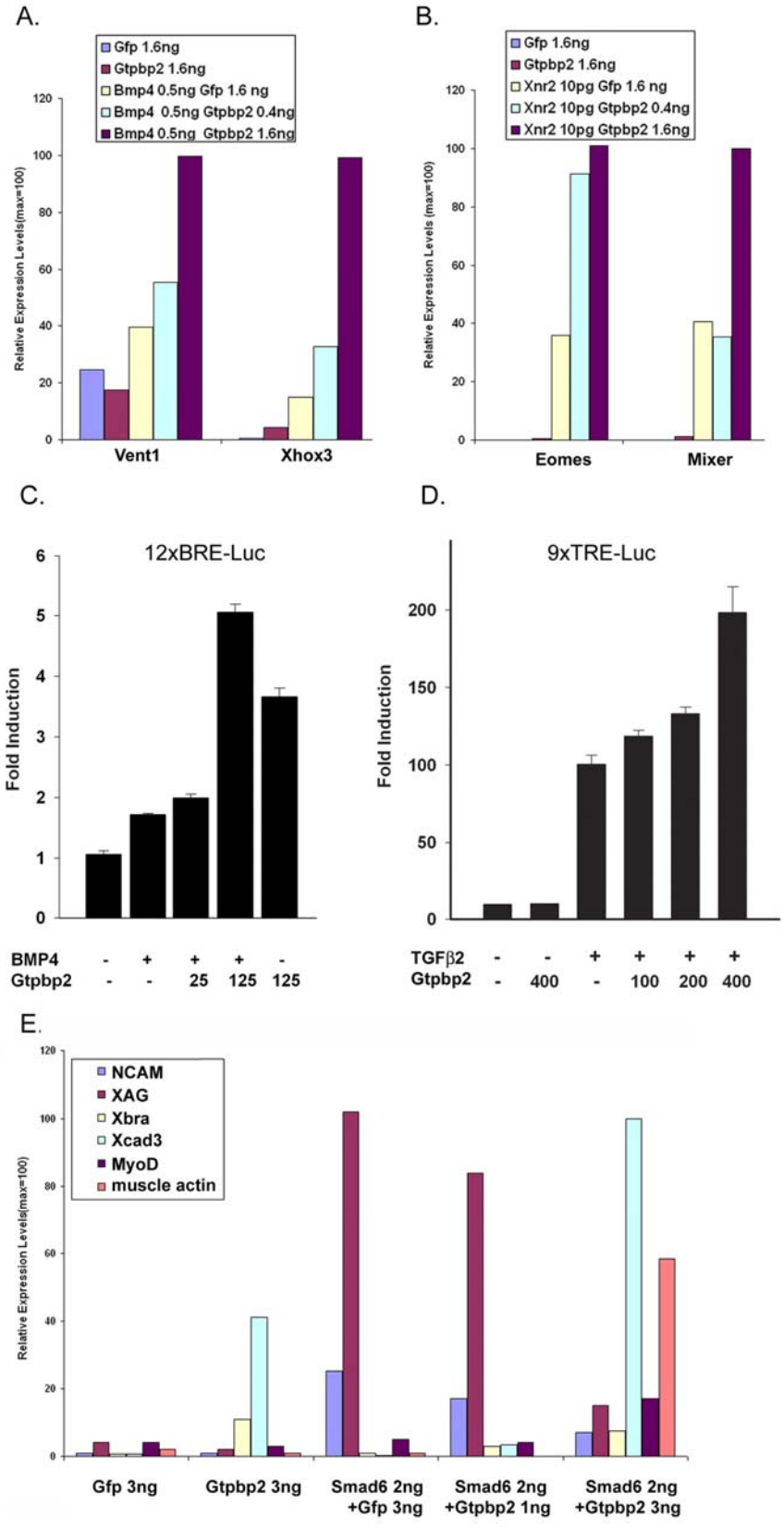

A.
Gfp 1.6ng
Gtpbp2 1.6ng
Bmp4 0.5ng Gfp 1.6 ng
Bmp4 0.5ng Gtpbp2 0.4ng
Bmp4 0.5ng Gtpbp2 1.6ng
Relative Expression Levels(max=100)
Vent1
Xhox3
B.
Gfp 1.6ng
Gtpbp2 1.6ng
Xnr2 10pg Gfp 1.6 ng
Xnr2 10pg Gtpbp2 0.4ng
Xnr2 10pg Gtpbp2 1.6ng
Relative Expression Levels (max=100)
Eomes
Mixer
C.
12xBRE-Luc
Fold Induction
BMP4 - + + + -
Gtpbp2 - - 25 125 125
D.
9xTRE-Luc
Fold Induction
TGFβ2 - - + + + +
Gtpbp2 - 400 - 100 200 400
E.
NCAM
XAG
Xbra
Xcad3
MyoD
muscle actin
Relative Expression Levels(max=100)
Gfp 3ng
Gtpbp2 3ng
Smad6 2ng +Gfp 3ng
Smad6 2ng +Gtpbp2 1ng
Smad6 2ng +Gtpbp2 3ng

Figure 3.5: GTPBP2 enhances BMP and TGF-β signaling.
A. GTPBP2 enhances BMP signaling in animal caps. Embryos injected with 500pg BMP4, and 400pg or 1.6ng GTPBP2, caps were cultured to stage 11, and Vent1, and Xhox3 genes analyzed with RT-PCR. **B.** GTPBP2 enhances Activin/Nodal signaling. Embryos injected with 10pg Xnr2, and increasing amounts of GTPBP2, gene induction assayed by RT-PCR. **C.** GTPBP2 induces luciferase activity from 12xBRE-Luc reporter. HepG2 cells were transfected with 25-125ng of Gtpbp2, and treated with 300ng/ml BMP4. **D.** GTPBP2 enhances luciferase activity from 9xTRE-Luc reporter. HepG2 cells tranfected with 100-400ng of GTPBP2, and treated with 5pg/ml TGF-β2. **E**. GTPBP2 reverses Smad6 mediated BMP inhibition and neurulation. Neural markers (NCAM, Xag), ventral-posterior mesoderm marker (xcad3), pan-mesodermal marker (Bracyhury), and dorsal mesoderm markers (MyoD, and muscle actin) were analyzed at stage 18 by RT-PCR.

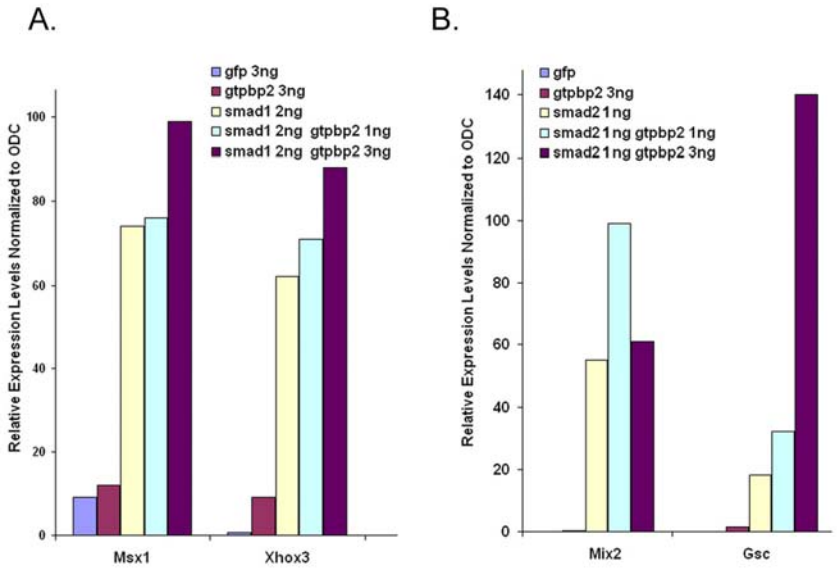


Figure 3.6: GTPBP2 enhances signaling when co-expressed with Smads.
A. GTPBP2 enhanced BMP signaling in animal caps when co-expressed with Smad1**. B.** GTPBP2 enhanced Nodal signaling when co-expressed with Smad2.

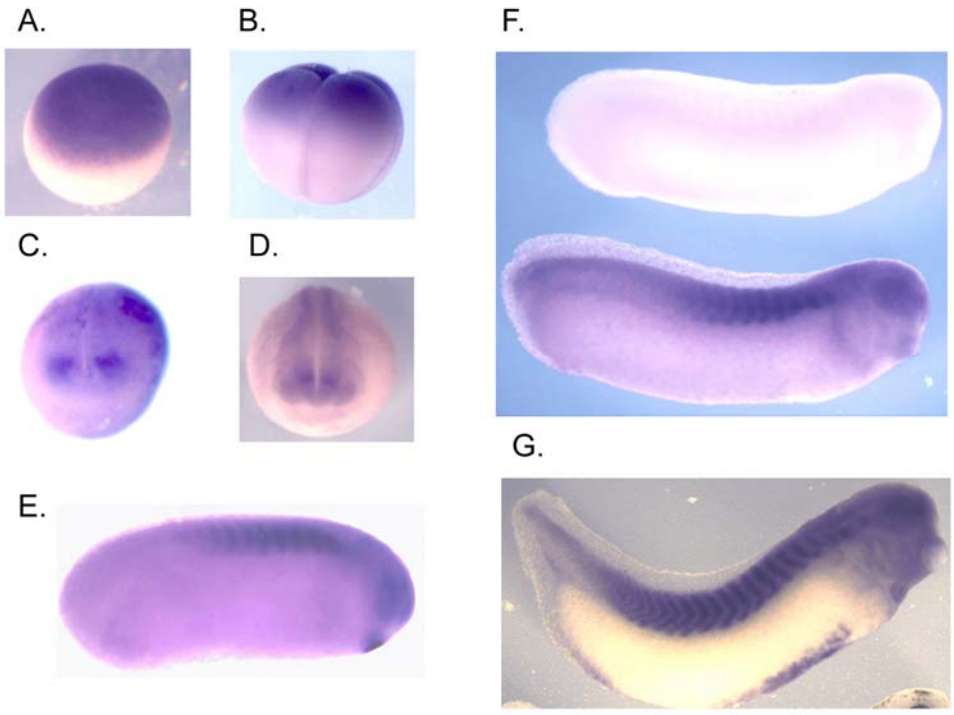


Figure 3.7: Whole mount in situ analysis of GTPBP2 at different developmental time points. Maternal GTPBP2 transcripts were detected at animal pole in blastula stages (**A, B**). In neurula stage GTPBP2 is expressed in anterior neural folds (**C, D**). In tadpole stages, expression is mainly in somites. **(E, F, G**) At stage 35, GTPBP2 is also expressed in ventral blood islands (VBI) (**G).**

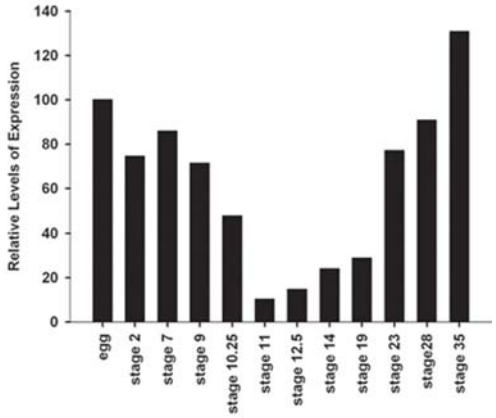


Figure 3.8:.Analysis of temporal expression pattern of GTPBP2 by semi-quantitative RT-PCR.

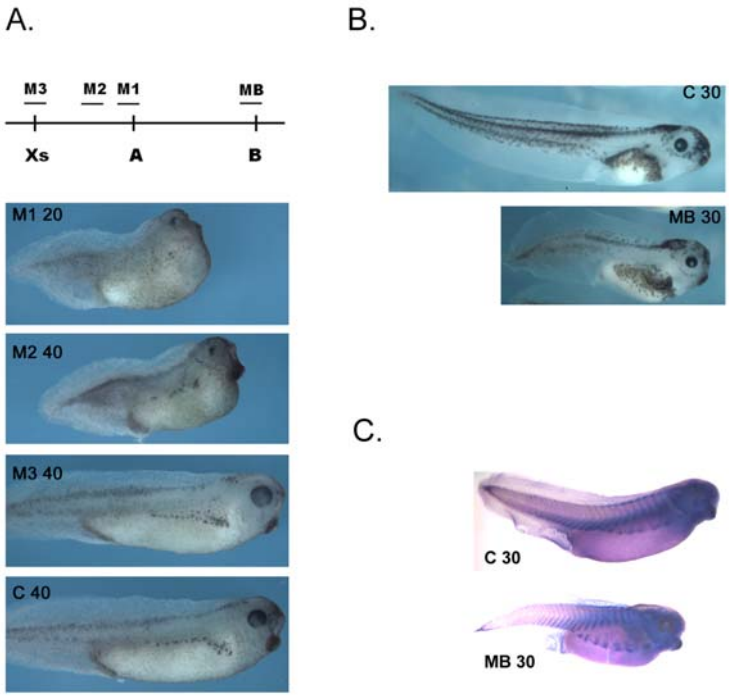


Figure 3.9: Effects of MOs designed against different potential start sites of GTPBP2.
A. Schematic representation of different MO's designed against GTPBP2. M1 and M2 morpholinos designed against GTPBP2A resulted in axial defects, M3 morpholino designed against putative GTPBP2A$_L$ did not cause embryonic defects. **B.** GTPBP2B specific MB morpholino caused shortened axis and robust somites at late tadpole stages. **C**. Somite staining was done with WISH using a MyoD probe.

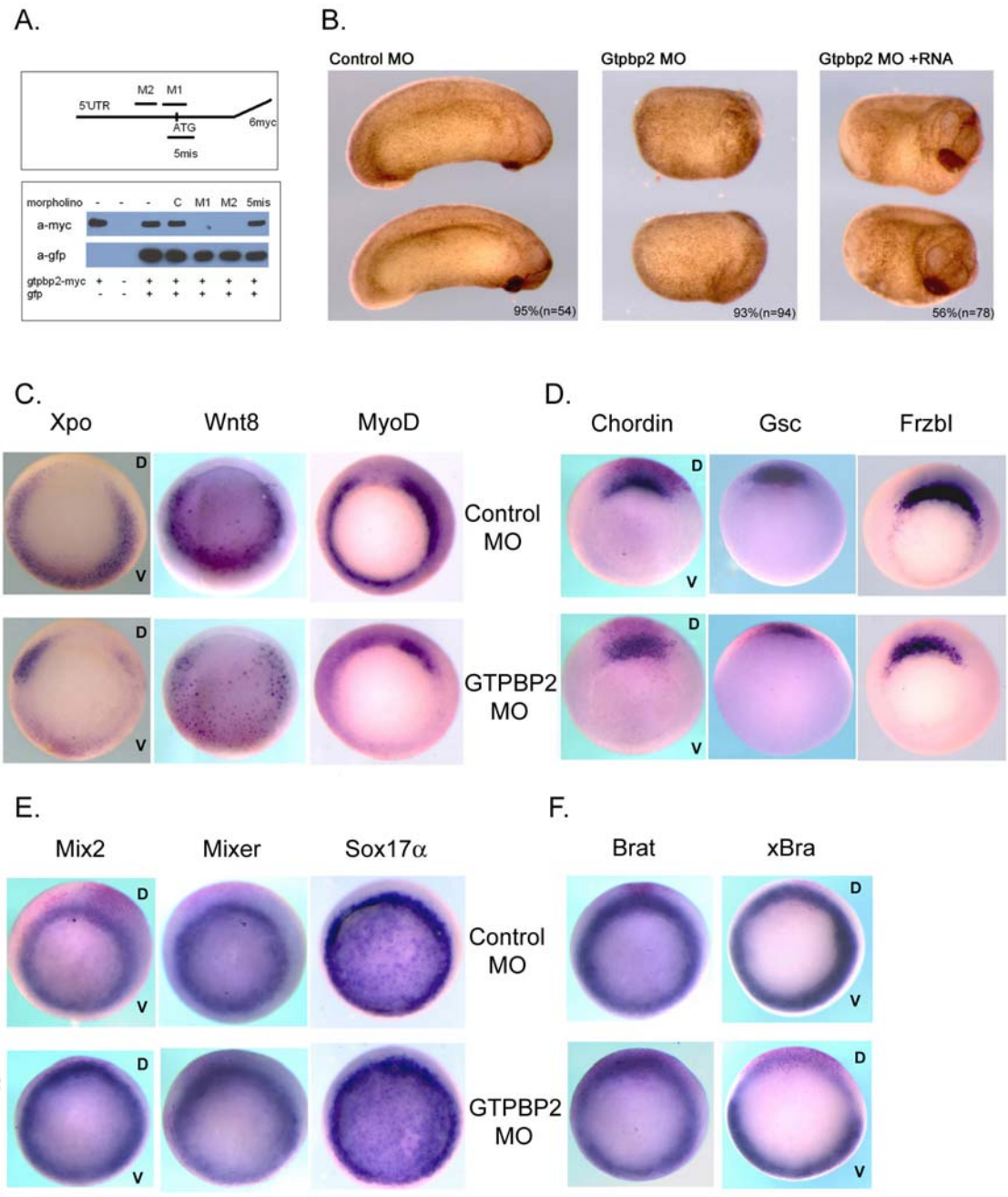

A.
M2
M1
5'UTR
ATG
6myc
5mis
morpholino - - - C M1 M2 5mis
a-myc
a-gfp
gtpbp2-myc + - + + + + +
gfp - - + + + + +
B.
Control MO
Gtpbp2 MO
Gtpbp2 MO +RNA
95%(n=54)
93%(n=94)
56%(n=78)
C.
Xpo
Wnt8
MyoD
Control MO
GTPBP2 MO
D
V
D.
Chordin
Gsc
Frzbl
E.
Mix2
Mixer
Sox17α
F.
Brat
xBra

Figure 3.10: Knockdown of GTPBP2 leads to severe axial patterning defects and disrupts dorsal ventral patterning of the mesoderm.

A. Western blot shows GTPBP2A morpholinos, M1 and M2 blocked translation from a C-terminally myc-tagged GTPBP2 construct in embryos. 1ng 5'UTR-GTPBP2-myc mRNA and 1ng GFP mRNA were co-injected with different morpholinos, and analyzed using a-myc and a-GFP antibodies at stage 11. **B.** GTPBP2 MO resulted in severe axial defects, including loss of all anterior structures and patterned somites. GTPBP2 MO phenotype was partially rescued by co-injection of a cocktail of GTPBP2A, and GTPBP2B mRNA. Embryos were injected dorsally at four-cell stage with 25ng of GTPBP2 MO, in rescue experiments MO was co-injected with 50pg of GTPBP2B, and 500pg of GTPBP2A mRNA. Rescue assays were done multiple times, numbers and pictures are from single experiment. In MO rescue assays, 56% of embryos co-injected with mRNA had head and anterior structures compared to 7% in Gtpbp2 MO injected samples. None of these samples looked wild type. **C.** GTPBP2 morphants exhibited severe reduction of ventra-lateral mesodermal markers, Xpo, Wnt8, and MyoD. Embryos were injected ventrally at 4-cell stage with 30ng Gtpbp2 MO, injected tissues traced with nuclear-β-galactosidase (Magenta-Gal was used as enzyme substrate), seen as pink dots. Embryos were oriented as dorsal being top. In situ hybridization assays were done three times with twenty embryos for each gene analyzed. Although there was variability in the extent of phenotype, none of the GTPBP2 morphant embryos showed wild type staining pattern. **D.** GTPBP2 knock-down did not affect expression of Mix2, Mixer, and Sox17a. **E.** Expression of organizer genes, Chordin, Gsc, and FrzbI were reduced in GTPBP2 morphants. **F.** Pan-mesodermal marker T-box gene Brachyury expression was severely reduced in GTPBP2 morphants. Experiments were repeated five times, with twenty embryos in each experiment. None of the GTPBP2 morphants showed wt type Brachyury expression pattern. Expression of another T-box gene Brat was not affected. Experiments were repeated three times for Brat, and genes in **C** and **D.** Morpholino was injected to dorsal blastomeres at four-cell stage in **D, E, and F.**

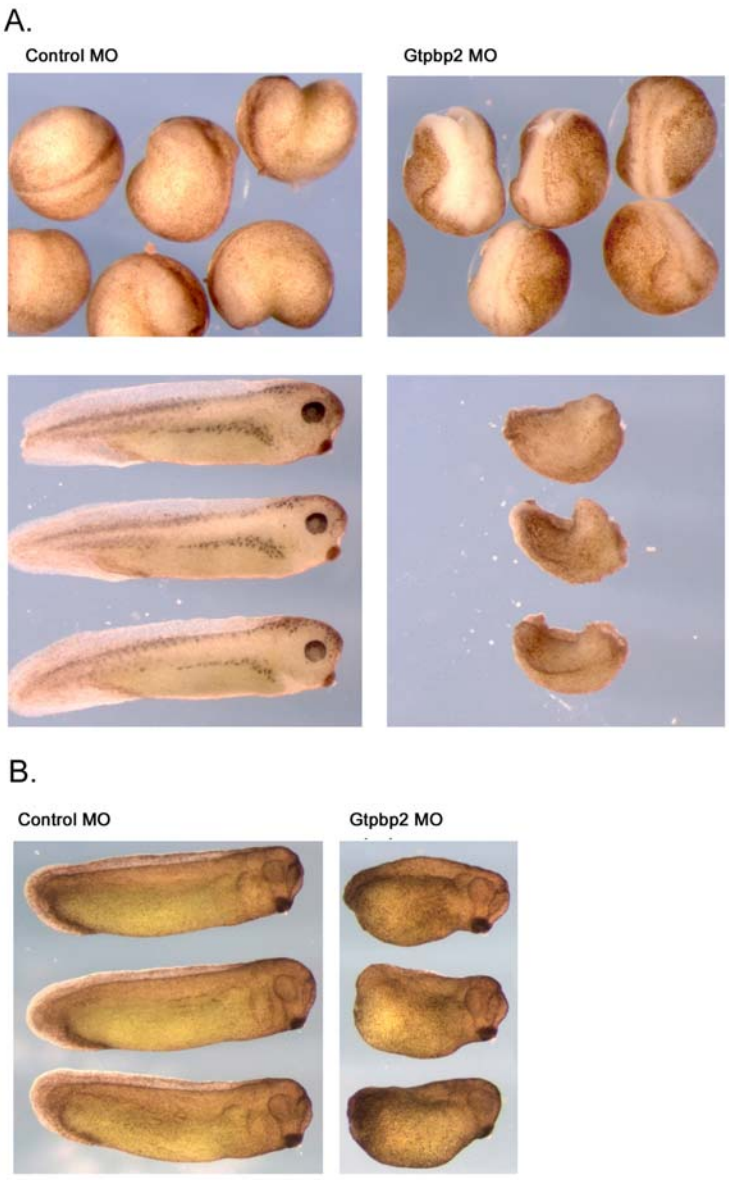


Figure 3.11: Knockdown of GTPBP2 leads to severe axial patterning defects.
A. Lateral injection of a total 30ng GTPBP2 MO at two-cell stage resulted in loss of all recognizable axial structures. Top panel shows neural folds form but did not fuse, leading embryos to look like they were stalled at early neurula stage. Embryos were pictured at stage 19. Lower panel shows embryos at stage 35. **B.** Embryos injected with 40ng GTPBP2 MO into two ventral blastomeres at four-cell stage did not develop tails and posterior somites, analyzed at stage 28.

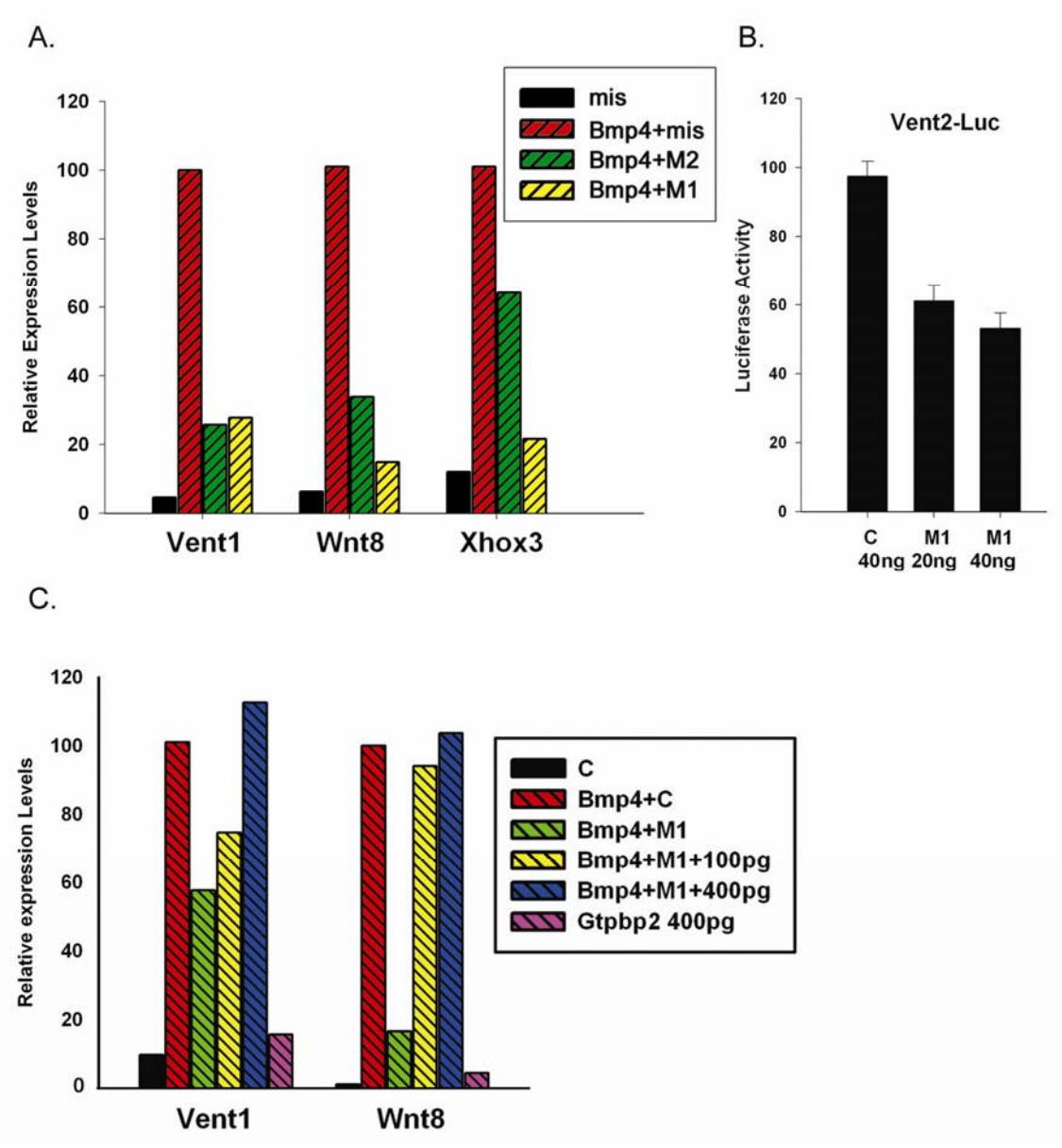


Figure 3.12: GTPBP2 is required for BMP signaling in animal cap explants
A. Loss of GTPBP2 inhibited BMP signaling. Embryos were injected at two-cell stage with 500pg BMP4 mRNA, and 20ng M1, or 40ng M2 morpholino. Animal cap tissue was dissected at Stage 8, and cultured to Stage 11. Bmp responsive genes were assayed by RT-PCR. **B.** GTPBP2 knock-down resulted in lower reporter activity from injected Vent2-Luciferase construct. Vent2 was a target of BMP signaling present at high levels in untreated animal caps. **E.** Co-injection of 100-400 ng of GTPBP2B mRNA with M1 morpholino restored BMP signaling.

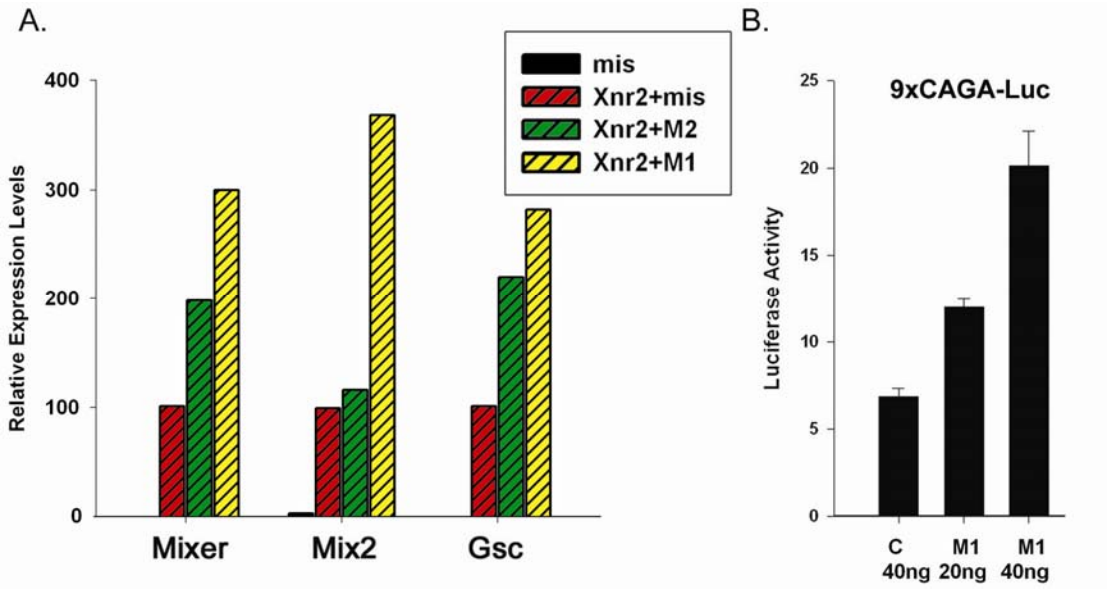


Figure 3.13: GTPBP2 inhibits Nodal signaling.
A. Xnr2 responsive genes were expressed at higher levels in GTPBP2 knock-down caps. Animal caps were treated with 10pg Xnr2. **B.** GTPBP2 knock-down embryos exhibited higher 9xCAGA-Luc reporter activity. 9xCAGA-Luc is a Smad3 activated synthetic reporter for TGF-β signaling (Dennler et al., 1998). Embryos were injected laterally at two-cell stage and assayed at stage 11.

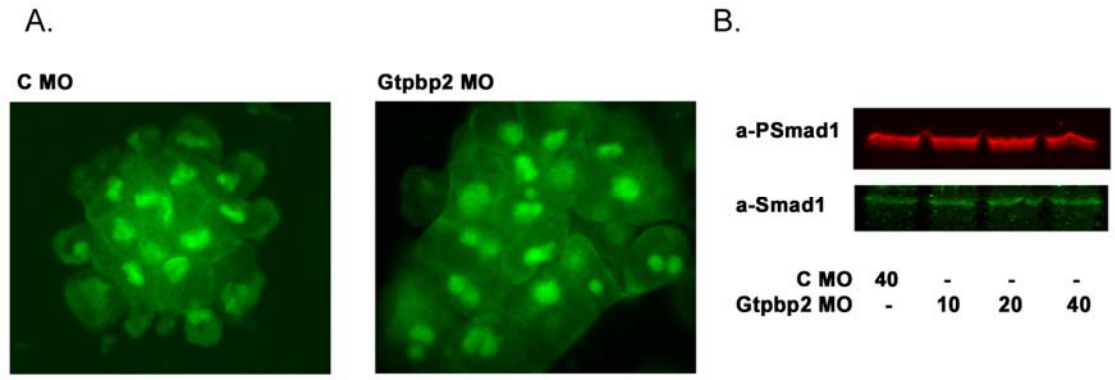


Figure 3.14: GTPBP2 knock-down does not affect Smad1 phoshorylation or localization.
A. Smad1 nuclear localization was not affected in dissociated animal cap cells cultured on fibronectin coated slides. Embryos were injected with GFP-Smad1 at two-cell stage. **B.** GTPBP2 knock-down did not affect P-Smad1 and Smad1 levels in animal caps. Embryos were injected with 500pg BMP4, animal cap tissue explanted at stage 8, and cultured to stage 11. Western Blotting was done using a-PSmad1 and a-Smad1 antibodies.

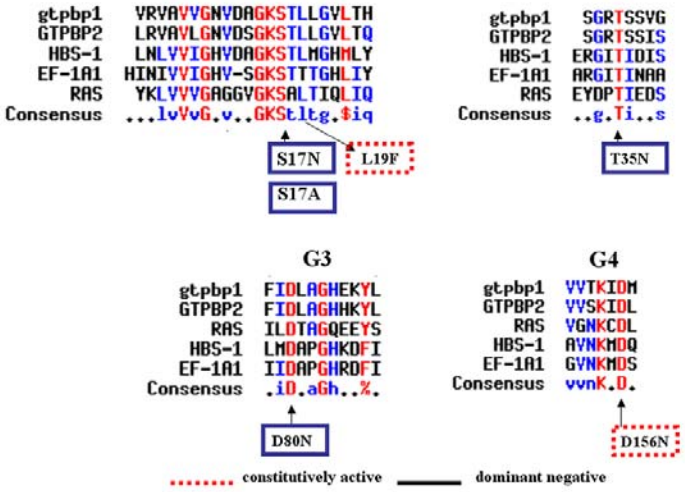


Figure 3.15: Schematic representation of GTPase motifs of GTPBP2. Mutations predicted to generate constitutively active mutants are shown in red and dominant negative mutants are shown in blue.

Chapter4: GTPBP1 interacts with Smads and is required for early development.

4.1 Introduction

GTPBP1 and GTPBP2 are highly homologous proteins which form a sub-family of GTPases phylogenetically distant from small GTPases (e.g. Ras, Rho) but closely related to translation factors (eEF1A, eRF3). GTPBP1 share 47% identity and 67% homology to GTPBP2, and 23% identity, and 45% homology to eEF1A. GTPBP1, like its homolog GTPBP2, contains an N-terminal GTPase domain, followed by two conserved C-terminal domains. All of these domains are conserved in eEF1A suggesting that GTPBP1 and GTPBP2 have a very similar three dimensional structure to eEF1A. The conserved C-terminal domains in eEF1A form a double barrel structure through which eEF1A binds to aa-tRNAs. Paralogs of eEF1A; eRF3 (GSPT1-2) and HBS1 have functionally diverged and do not bind aa-tRNA (Inagaki et al., 2003). In addition to its role in translation termination, eRF3 is involved in mRNA deadenylation and degradation (Funakoshi et al., 2007). And HBS1 involved in a quality control system (No-Go decay) that recognizes and degrades non-functional mRNAs (Doma and Parker, 2006). Based on high homology between GTPBPs and eEF1A, and its paralogs, it is reasonable to suggest that GTPBP1 and GTPBP2 bind to some form of RNA or possibly to DNA.

GTPBP1 was first isolated as an interferon-γ inducible gene in monocytes (Senju and Nishimura, 1997). Nishimura lab generated GTPBP1 deficient mice to address the physiological role of GTPBP1 (Senju et al., 2000). The mutant mice were born at the expected mendelian frequency, developed normally, and were fertile. They investigated the tissue specific expression of GTPBP1 protein and found that it is expressed in

neurons, smooth muscle, and monocytes in adult mice. There were no abnormalities in immune responses generated by monocytes either. The absence of a clear phenotype in GTPBP1 deficient mice was explained by the presence of GTPBP2, which may be compensating functionally for GTPBP2.

In previous chapters, I described that GTPBP2 binds to Smads, and acts as a positive regulator of TGF-β signaling. High similarity between GTPBP1 and GTPBP2 suggests that GTPBP1 may bind to Smads as well. Here, I show GTPBP1 binds to Smad1 and Smad3. GTPBP1 overexpression does not cause a phenotype in embryos. However, GTPBP1 is required for embryonic development, and GTPBP1 morphants show severe dorsal anterior defects.

4.2 Results

4.2.1 GTPBP1 interacts with Smad1 and Smad3

To determine whether GTPBP1 binds to Smads like its close homolog GTPBP2, I performed co-immunoprecipitation assays in Hek293T cells overexpressing GTPBP1 and major R-Smads (Smads 1, 2 and 3), co-Smad (Smad4), and I-Smads (Smad6 and 7). Flag-tagged versions of the Smads were immunoprecipitated and tested for binding with HA-GTPBP1 by western blot. I found that GTPBP2 can interact with Smad1 and Smad3 (Figure 4.1).

4.2.2 GTPBP1 morpholino causes dorso-anterior defects.

I began investigating the function of GTPBP1 by testing whether it had any effects on Xenopus embryos when ectopically expressed. However, overexpressed GTPBP1 did not cause any phenotype. To address if GTPBP1 is required for Xenopus development I performed protein knock-down experiments by utilizing translation-blocking antisense morpholino oligos (MOs). When I injected 20ng of GTPBP1 MO to two-cell stage embryos targeting all tissues, embryos dissociate after completing gastrulation (Figure 4.2A). Therefore it seems that GTPBP1 is required for embryonic survival. To identify tissues that are affected first, I injected MO at lower doses. At 15ng MO injections, many of the MO treated embryos survive albeit head loss and severe dorso-anterior defects (Figure 4.2B). And At 10ng MO injection, embryos have smaller heads and shorter axis when injected dorsally (Figure 4.2C), and abnormalities in gut formation when injected ventrally (Figure 4.2D).

4.3 Discussion

GTPBP1 and GTPBP2 are close homologs. Therefore, I hypothesized that GTPBP1 would bind Smads. Indeed, GTPBP1 binds to Smad1 and Smad3. However, there are differences between GTPBP1 and GTPBP2 in terms of Smad binding specificity. In addition to Smad1 and Smad3, GTPBP2 also binds to Smad4 and Smad6. Also GTPBP2 binding to Smad3 is stronger than GTPBP1. Furthermore, in in-vitro interaction assays, I did not observe an interaction between GTPBP1 and Smad1. One explanation for this discrepancy is that binding of GTPBP1 to Smads may be ligand dependent and

regulated. It is also possible that GTPBP1 does not directly bind Smads but co-immunoprecipitates indirectly binding to other Smad binding proteins.

My initial analysis shows that GTPBP1 is essential for survival of the embryos. Injection of GTPBP1 morpholino results in cell dissociation from neural folds at the end of gastrulation, and eventually death of the emrbyos. When injected ventrally, I observed similar defects; embryos collapsed and dissociated at mid-neurula stages starting from targeted ventral tissues. Embryos survived better until tadpole stages when MO levels were decreased to 10ng. These embryos had smaller heads, and were missing anterior somites. Similarly, when injected ventrally, organization of ventral tissues were disrupted. Hence, I concluded that GTPBP1 is essential protein for cell metabolism.

In a previous published study, Gtpbp1 gene was "disrupted" to generate GTPBP1 deficient mice. The mutant mice were born at the expected mendelian frequency, developed normally, and were fertile. Authors of this study explained the lack of any phenotype in GTPBP1 deficient mice by the presence of Gtpbp2 (Senju et al., 2000). Although the genetic disruption of Gtpbp1 locus was well characterized in that paper, it is important to mention that the exons deleted in the knock-out mouse were not the first two exons of the gene as described but rather code for internal aa's. Gtpbp1 has a similar genomic structure to Gtpbp2, in a way the real first two exons coding for the long form of the protein are located far upstream, leading these researchers to assume that they have full genomic locus characterized (Figure 2.2). In addition to this, Western Blot analysis to assess the protein knock-out was done by an antibody that was generated against the exons, which were removed in the knockout. The authors used a second antibody specific to C terminal end of the protein, but in this experiment there was a second smaller band

present in both lanes. Therefore, it is noteworthy to examine the presence of Gtpbp1 protein using antibodies generated against conserved elongation factor domains to prove that Gtpbp1 activity is completely destroyed.

4.4. Materials and Methods

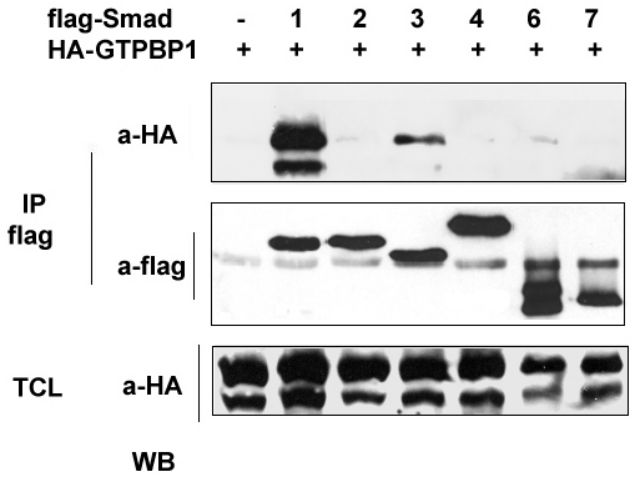
4.4.1. Co-Immunoprecipitation of GTPBP1 and Smads

I obstained a cDNA clone coding for GTPBP1 (IMAGE: 3403772) from ResGen, and then sub-cloned it into pCS2-3xHA for co-immunoprecipitation assay. HA-GTPBP1 and flag-Smads were overexpressed in Hek293T cells using transfection reagent Fugene6 (Roche) and were grown in 10% calf serum (Hyclone)/ 45% F12 / 45% DMEM (Gibco). Cells were lysed 24 hours after transfection with PBS containing 1% Triton X-100, 2mM EDTA, 1mM Na_3VO_4 and complete protease inhibitors (Roche). To pull down Flag-Smads, anti-Flag M2 agarose (Sigma) beads were incubated with lysates for 1 hour at 4C. Beads were spun and washed with cold lysis buffer several times, and SDS sample buffer was added to the beads and proteins were resolved by SDS-PAGE. Anti-HA-HRP (Roche) (1:500) and anti-Flag M2 (Sigma) (1:2000) followed by anti-mouse-conjugated HRP (Sigma) (1:5000) were used to detect HA-GTPBP1 and Flag-Smads, respectively.

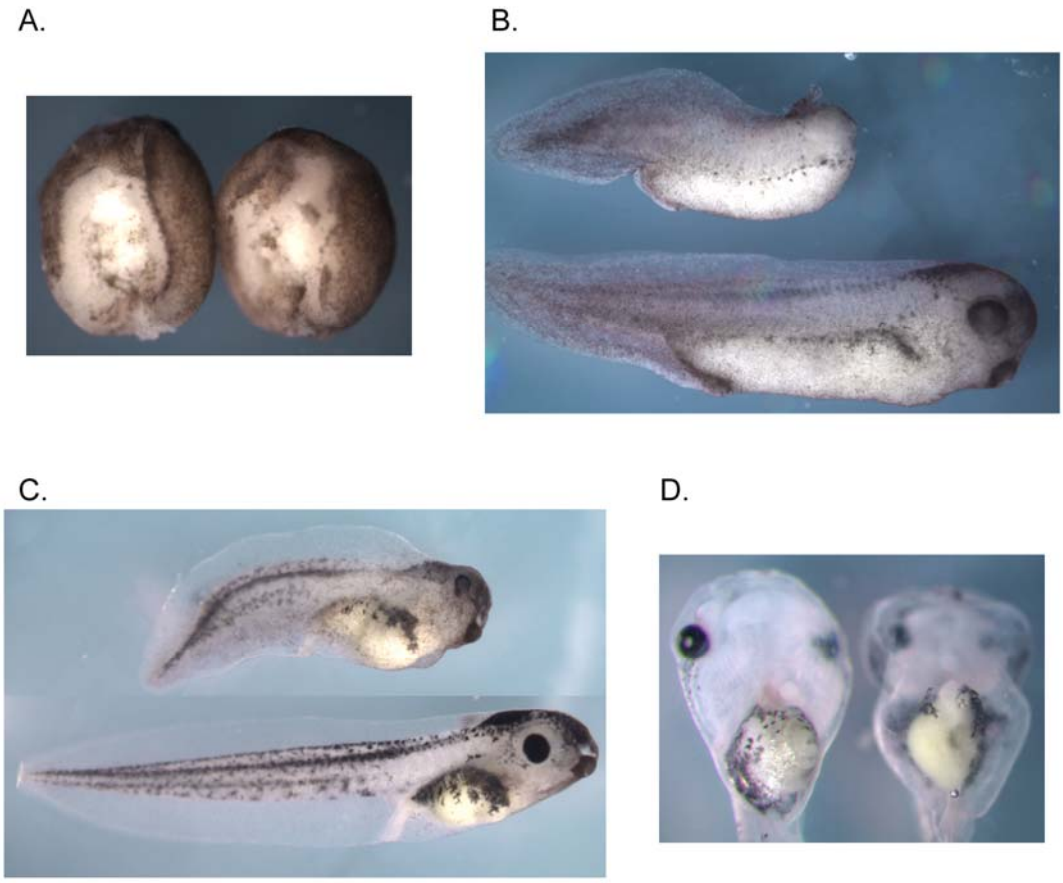
4.4.2. Embryo manipulation with mRNA and morpholino

Collection of Xenopus embryos and microinjection was done as described previously (Alexandrova and Thomsen, 2006). Anti-sense morpholino for GTPBP1 (^{5}GCCCTCATCCATCCGGTCCCGCAAC^3) was obtained from Gene-Tools, scrambled morpholino from was used as control.

4.5 Figures



4.1: GTPBP1 interacts with Smad1 and Smad3. Flag-Smad constructs were co-expressed with HA-GTPBP1. Cell lysated were co-immunoprecipitated using Flag-agarose beads, and analyzed by WB using α-HA-HRP, and α-F M2 antibodies. Lower panel shows total cell lysate GTPBP1 levels, top two panels show immuno-precipitated proteins.



4.2: GTPBP1 is required for development and survival. **A.** Injection of 20ng GTPBP1 MO at 2-cell stage to both blastomeres resulted in dissociation and death of embryos. **B.** Injection of 15ng GTPBP2 morpholino to DMZ lead to the loss of dorsal structures.**C.** GTPBP2 MO at 10ng caused shortened axis and small head. **D.** Injection of GTPBP1 morpholino to VMZ resulted abnormalities in gut formation.

Chapter 5: Future Directions

We started this project by identifying GTPBP2 as a potential Smad1 binding protein from a yeast two hybrid screen. I have confirmed this interaction and showed that GTPBP2 binds R-Smads (Smad1 and Smad3), co-smad (Smad4), and I-Smad (Smad6). Overexpression experiments in embryos and cultured cells suggested that GTPBP2 is a positive regulator of TGF-β signaling pathways. In vivo analysis of GTPBP2 function by morpholino knock-down in Xenopus embryos proved that GTPBP2 is required for BMP signaling in embryonic development. Although I have answered many crucial questions about the nature of GTPBP2-Smad interaction, these only correspond to the tip of the iceberg and I still only have speculations about the biochemical function of GTPBP2.

Since there is no literature on biochemical activities and function of GTPBP2, focused my experiments to testing the effect of GTPBP2 on TGF-β/Smad signaling with a hypothesis of GTPBP2 being a regulator of TGF-β signaling. Smad proteins are transcription factors and function in the nucleus to activate gene expression. I found that GTPBP2 is a nuclear protein and is required for proper signaling through Smad proteins. EEF1A, a close homolog of GTPBP2, is shown to activate HSF-1 transcription factor by forming a ribonucleoprotein complex with an untranslated RNA, HSR-1, and promoting the trimerization of HSF-1 (Shamovsky et al., 2006). Hence I predict that GTPBP2 may be an activator of Smad signaling complexes in a similar fashion. First, GTPBP2 may be required for R-Smad/co-Smad binding, and this will be tested by assessing the effect of GTPBP2 knock-down on R-Smad/co-Smad complexes in embryos. Alternatively, GTPBP2 may not be necessary for R-Smad/co-Smad binding but required for a Smad

mega-complex including other transcription and processivity factors required for in vivo DNA binding and transcriptional fidelity. I will test this possibility by utilizing CHIP methods, and probe the level of endogenous promoters such as Vent2 and Id3 that are bound to Smad proteins in GTPBP2 knock-down embryos or cell lines.

Although I tested the effects of GTPBP2 on TGF-β/Smad signaling at transcriptional level, there is new evidence showing that Smads are also involved in regulating post-transcriptional processes. Specifically, Smads bind to a subunit of DROSHA mi-RNA processing complex and possibly to pri-mi-RNA, and control microRNA maturation (Davis et al., 2008). This interaction is mediated through MH1 domain of Smad1. The MH1 domain of R-Smads binds to DNA by specifically recognizing a sequence element (Massague et al., 2005). It is speculated that the Smad MH1 domain may recognize an RNA sequence element, and thus provide specificity in the selection of TGF-β target miRNA (Davis et al., 2008). Smad are also shown to interact with many proteins that are functionally or structurally related to mRNA stability, splicing, or has RNA binding domains such as Tob, Sf3b2, and xMAN1 (Xiong et al., 2006; Yoshida et al., 2000; Warner et al., 2003; Osada et al., 2003; Ishimura et al., 2006; also see Chapter 3 for details). In a search for GTPBP2 binding proteins that may provide us clues where and how GTPBP2 functions, I found that worm homolog of Sf3b2 interacts with GTPBP2. I confirmed the interaction between xSf3b2 and xGTPBP2 (Figure 5.1). These studies suggest that Smad mediated post-transcriptional regulation may not be limited to microRNA processing but include regulation of mRNA stability or splicing. Indeed I have clues pointing out to such mechanism. In Xenopus embryos and animal cap explants, GTPBP2 enhances BMP and TGF-β signaling. However, when

GTPBP2 is knocked-down, I observed that, although GTPBP2 is required for BMP signaling (Figure 3.12) as expected, loss of GTPBP2 increased the response to nodal ligands in caps, and similarly I observed increased reporter activity in embryos (Figure 3.13). Interestingly, despite enhancing TGF-β signaling at RNA levels (Figure 3.5B-D, 3.6), GTPBP2 inhibits reporter activity from a nodal responsive endogenous promoter Zic3 (Figure 5.2), as well as synthetic 9xCAGA-Luc construct (Figure 5.3A-B) in animal cap explants and embryos. If GTPBP2 acts only at transcriptional level, then I would expect to see the same behavior at RNA and protein levels. Alternatively, if GTPBP2 is a general regulator of mRNA stability via sequestering Smads from Tob binding and possibly protecting TGF-β target genes, then I should expect to see stabilized mRNA levels and similarly more protein translated. If GTPBP2 acts in microRNA processing together with Smads, then GTPBP2 should be involved in production of mi-RNAs that would block translation of TGF-β responsive genes but promote BMP signaling. MicroRNAs can direct the RISC to downregulate gene expression by either of two posttranscriptional mechanisms: mRNA cleavage or translational repression by binding to 3'UTR (Bartel et al., 2004). Since reporter constructs used in my experiments do not contain endogenous UTR sequences for any gene of interest it is unlikely that TGF-β specific micro-RNAs would bind to these mRNAs to inhibit their translation. Any of these scenarios could explain the role of GTPBP2 in BMP signaling but none of them explains the effects on Nodal responsive RNA and reporter levels in embryos. To understand this contradictory behavior on Nodal target genes in Xenopus, one should first understand how BMP signaling is positively regulated by GTPBP2. I proposed experiments to test its role in transcription above. If these experiments prove that

GTPBP2 is not involved at transcriptional level, then I should first test the effects of GTPBP2 on mRNA stability and or on Tob activity.

GTPBP2 is predicted to function as a GTPase by its high homology to other GTPases. However, I did not test this aspect in detail except my attempts to make dominant acting mutants. Although GTPase domain is not necessary for Smad binding, it is reasonable to think that the activity of GTPBP2 is dependent on its GTP/GDP state. GTPases are regulated by upstream signals through Guanine Exchange Factors and GTPase Activating Proteins which modulate the rate of GTP hydrolysis and hence the level of activity (Sprang, 1997; Vetter and Wittinghofer, 2001). It is highly probable that GTPBP2 is modulated by such regulatory mechanism. Therefore, it is an open question if and how GTPBP2 is regulated by extracellular stimuli that may be critical for Smad activation by GTPBP2. Of further consideration, GTPBP2B is a nuclear protein in all cell types tested whereas GTPBP2A is localized to cytoplasm in most cell types tested. Therefore, it is possible that the N terminal region of GTPBP2 is regulated by a signal that controls nucleocytoplasmic shuttling of GTPBP2. Also GTPBP2B may be a form that does not require this signal to activate TGF-β/Smad signaling in the nucleus.

GTPBP2 mRNA is present maternally and shows a dynamic pattern during development. GTPBP2 is localized to somites, heart and blood islands in tadpole stages. In my loss of function experiments, since I observed defects early in gastrulation stage, I focused on explaining these defects and the role of GTPBP2 in early BMP signaling. However, it is very likely that GTPBP2 is involved in somitogenesis, heart and blood development. GTPBP1 was also first isolated as an induced gene in a monocyte cell line, and used as blood lineage marker in zebrafish development (Senju et al., 1997; Galloway

et al., 2005). Since GTPBP2 transcripts are maternally present, one way of avoiding early defects would be to target zygotic transcripts with a splicing blocking morpholino. I do not know anything about endogenous protein levels of GTPBP2, and its possible forms. I attempted such studies by purchasing two commercial antibodies, and by contracting a company to raise another one, all without success. This limited my ability to assess the effects of MOs on endogenous protein levels as well as the ability to do understand the endogenous interactions between Smads and GTPBP2. Therefore, it is utmost priority to generate a frog specific GTPBP2 antibody, to use to understand mechanisms of endogenous protein function in the future.

5.1 Figures

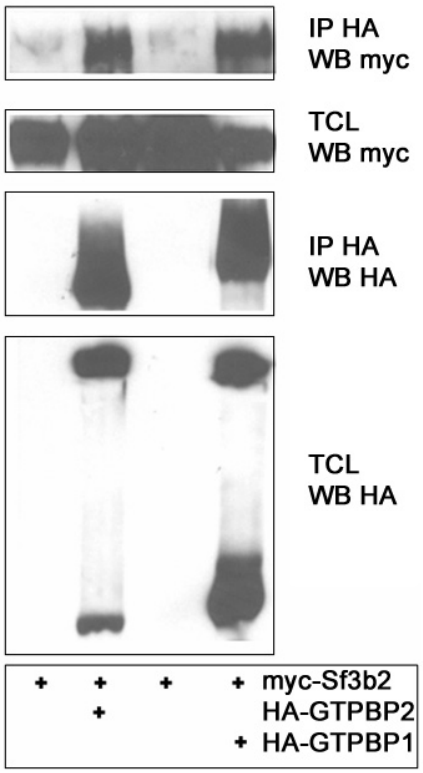


Figure 5.1: Sf3b2 interacts with GTPBP1 and GTPBP2. myc-Sf3b2 and HA-GTPBPs were expressed in Hek293T cells, and coimmunoprecipitated using polyclonal rabbit-anti-HA antibodes (Santa Cruz). Proteins were detected using HA-HRP and myc-HRP antibodies (Roche).

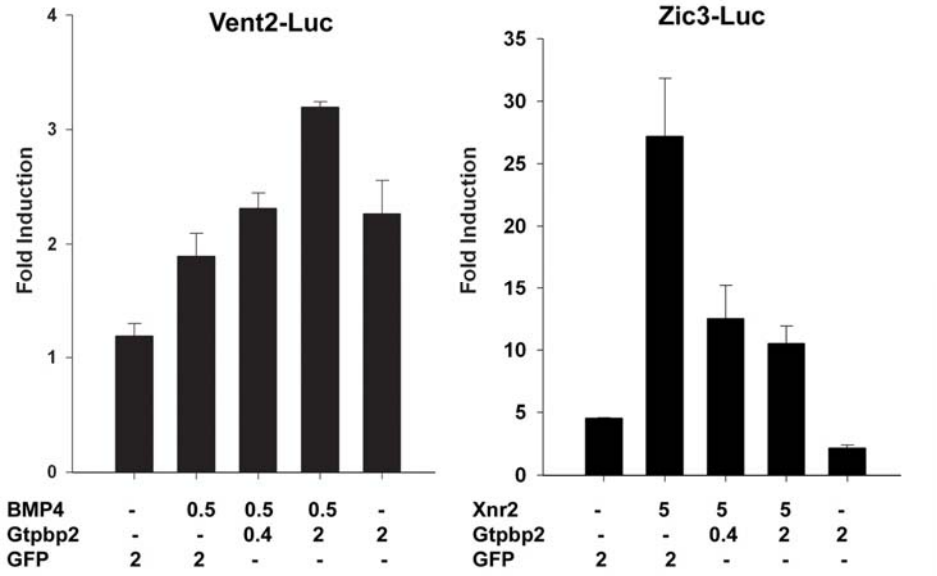


Figure 5.2: The activity from a BMP responsive Vent2-Luc reporter construct is enhanced by GTPBP2, whereas the activity from a nodal responsive Zic3-Luc reporter is inhibited. Embryos were injected at two cell stage; caps were excised at stage 8, and cultured to stage11.

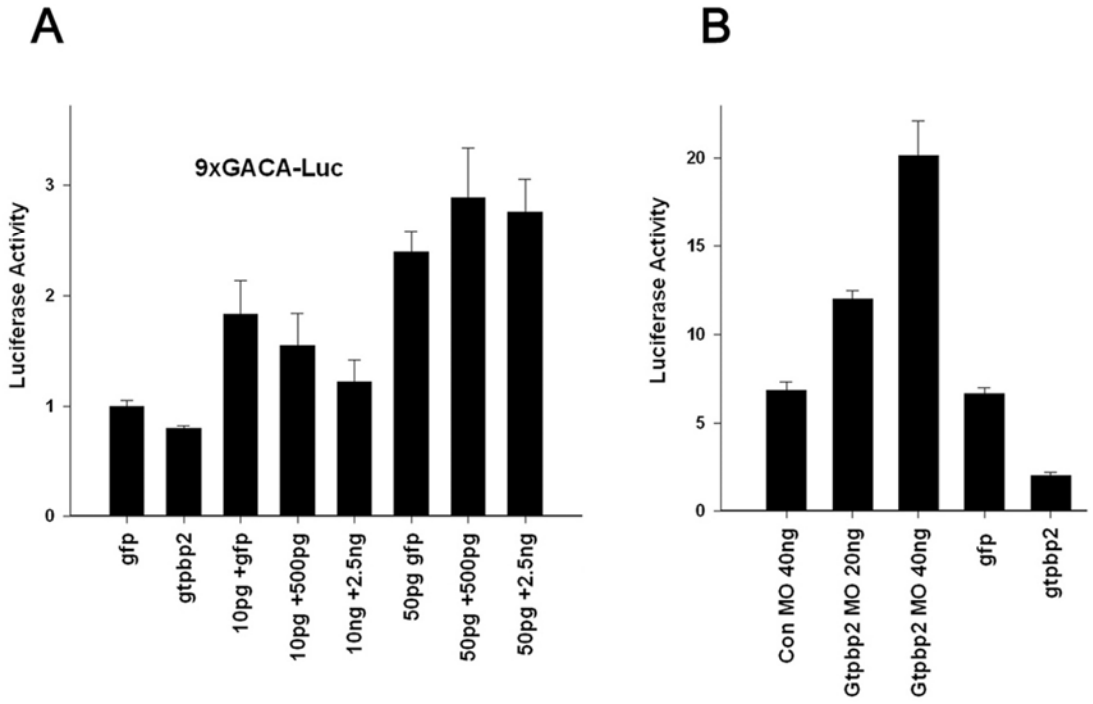


Figure 5.3 GTPBP2 inhibits the luciferase activity from synthetic reporter construct, 9xCAGA-Luc. A. in cap explants B. in embryos

Bibliography

Agius, E., Oelgeschlager, M., Wessely, O., Kemp, C., and De Robertis, E. M. (2000). Endodermal Nodal-related signals and mesoderm induction in Xenopus. Development *127*, 1173-1183.
Akiyoshi, S., Inoue, H., Hanai, J., Kusanagi, K., Nemoto, N., Miyazono, K., and Kawabata, M. (1999). c-Ski acts as a transcriptional co-repressor in transforming growth factor-beta signaling through interaction with smads. J Biol Chem *274*, 35269-35277.
Alexandrova, E. M., and Thomsen, G. H. (2006)**.** Smurf1 regulates neural patterning and folding in Xenopus embryos by antagonizing the BMP/Smad1 pathway. Dev Biol *299*, 398-410.
Amaya, E., Stein, P. A., Musci, T. J., and Kirschner, M. W. (1993). FGF signalling in the early specification of mesoderm in Xenopus. Development *118*, 477-487.
Annes, J. P., Munger, J. S., and Rifkin, D. B. (2003). Making sense of latent TGFbeta activation. J Cell Sci *116*, 217-224.
Attisano, L., and Wrana, J. L. (2002). Signal transduction by the TGF-beta superfamily. Science *296*, 1646-1647.
Bai, S., and Cao, X. (2002). A nuclear antagonistic mechanism of inhibitory Smads in transforming growth factor-beta signaling. J Biol Chem *277*, 4176-4182.
Bai, Y., Yang, C., Hu, K., Elly, C., and Liu, Y. C. (2004). Itch E3 ligase-mediated regulation of TGF-beta signaling by modulating smad2 phosphorylation. Mol Cell *15*, 825-831.
Baker, J. C., and Harland, R. M. (1996). A novel mesoderm inducer, Madr2, functions in the activin signal transduction pathway. Genes Dev *10*, 1880-1889.
Bartel, D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell *116*, 281-297.
Bengtsson, L. (2007). What MAN1 does to the Smads. TGFbeta/BMP signaling and the nuclear envelope. Febs J *274*, 1374-1382.
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.
Bouwmeester, T. (2001). The Spemann-Mangold organizer: the control of fate specification and morphogenetic rearrangements during gastrulation in Xenopus. Int J Dev Biol *45*, 251-258.
Bouwmeester, T., Kim, S., Sasai, Y., Lu, B., and De Robertis, E. M. (1996). Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. Nature *382*, 595-601.
Chacko, B. M., Qin, B. Y., Tiwari, A., Shi, G., Lam, S., Hayward, L. J., De Caestecker, M., and Lin, K. (2004). Structural basis of heteromeric smad protein assembly in TGF-beta signaling. Mol Cell *15*, 813-823.
Chang, C., Brivanlou, A. H., and Harland, R. M. (2006). Function of the two Xenopus smad4s in early frog development. J Biol Chem *281*, 30794-30803.
Chang, C., and Harland, R. M. (2007). Neural induction requires continued suppression of both Smad1 and Smad2 signals during gastrulation. Development

134, 3861-3872.
Chang, C. C., Lin, D. Y., Fang, H. I., Chen, R. H., and Shih, H. M. (2005). Daxx mediates the small ubiquitin-like modifier-dependent transcriptional repression of Smad4. J Biol Chem *280*, 10164-10173.
Chang, J. S., Seok, H., Kwon, T. K., Min, D. S., Ahn, B. H., Lee, Y. H., Suh, J. W., Kim, J. W., Iwashita, S., Omori, A. (2002). Interaction of elongation factor-1alpha and pleckstrin homology domain of phospholipase C-gamma 1 with activating its activity. J Biol Chem *277*, 19697-19702.
Chen, H. B., Shen, J., Ip, Y. T., and Xu, L. (2006). Identification of phosphatases for Smad in the BMP/DPP pathway. Genes Dev *20*, 648-653.
Chen, X., Weisberg, E., Fridmacher, V., Watanabe, M., Naco, G., and Whitman, M. (1997). Smad4 and FAST-1 in the assembly of activin-responsive factor. Nature *389*, 85-89.
Cornell, R. A., and Kimelman, D. (1994). Activin-mediated mesoderm induction requires FGF. Development *120*, 453-462.
Crease, D. J., Dyson, S., and Gurdon, J. B. (1998). Cooperation between the activin and Wnt pathways in the spatial control of organizer gene expression. Proc Natl Acad Sci U S A *95*, 4398-4403.
Dale, L., and Jones, C. M. (1999). BMP signalling in early Xenopus development. Bioessays *21*, 751-760.
Dal-Pra, S., Furthauer, M., Van-Celst, J., Thisse, B., and Thisse, C. (2006). Noggin1 and Follistatin-like2 function redundantly to Chordin to antagonize BMP activity. Dev Biol *298*, 514-526.
Davis, B. N., Hilyard, A. C., Lagna, G., and Hata, A. (2008). SMAD proteins control DROSHA-mediated microRNA maturation. Nature *454*, 56-61.
De Robertis, E. M., and Kuroda, H. (2004). Dorsal-ventral patterning and neural induction in Xenopus embryos. Annu Rev Cell Dev Biol *20*, 285-308.
Delaune, E., Lemaire, P., and Kodjabachian, L. (2005). Neural induction in Xenopus requires early FGF signalling in addition to BMP inhibition. Development *132*, 299-310.
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*, 3091-3100.
Di Guglielmo, G. M., Le Roy, C., Goodfellow, A. F., and Wrana, J. L. (2003). Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover. Nat Cell Biol *5*, 410-421.
Doma, M. K., and Parker, R. (2006). Endonucleolytic cleavage of eukaryotic mRNAs with stalls in translation elongation. Nature *440*, 561-564.
Dosch, R., Gawantka, V., Delius, H., Blumenstock, C., and Niehrs, C. (1997). Bmp-4 acts as a morphogen in dorsoventral mesoderm patterning in Xenopus. Development *124*, 2325-2334.
Dosch, R., and Niehrs, C. (2000). Requirement for anti-dorsalizing morphogenetic protein in organizer patterning. Mech Dev *90*, 195-203.
Duan, X., Liang, Y. Y., Feng, X. H., and Lin, X. (2006). Protein serine/threonine phosphatase PPM1A dephosphorylates Smad1 in the bone morphogenetic

protein signaling pathway. J Biol Chem *281*, 36526-36532.
Dunn, N. R., Vincent, S. D., Oxburgh, L., Robertson, E. J., and Bikoff, E. K. (2004). Combinatorial activities of Smad2 and Smad3 regulate mesoderm formation and patterning in the mouse embryo. Development *131*, 1717-1728.
Dupont, S., Zacchigna, L., Cordenonsi, M., Soligo, S., Adorno, M., Rugge, M., and Piccolo, S. (2005). Germ-layer specification and control of cell growth by Ectodermin, a Smad4 ubiquitin ligase. Cell *121*, 87-99.
Ebisawa, T., Fukuchi, M., Murakami, G., Chiba, T., Tanaka, K., Imamura, T., and Miyazono, K. (2001). Smurf1 interacts with transforming growth factor-beta type I receptor through Smad7 and induces receptor degradation. J Biol Chem *276*, 12477-12480.
Eisen, J. S., and Smith, J. C. (2008). Controlling morpholino experiments: don't stop making antisense. Development *135*, 1735-1743.
Ezzeddine, N., Chang, T. C., Zhu, W., Yamashita, A., Chen, C. Y., Zhong, Z., Yamashita, Y., Zheng, D., and Shyu, A. B. (2007). Human TOB, an antiproliferative transcription factor, is a poly(A)-binding protein-dependent positive regulator of cytoplasmic mRNA deadenylation. Mol Cell Biol *27*, 7791-7801.
Feledy, J. A., Beanan, M. J., Sandoval, J. J., Goodrich, J. S., Lim, J. H., Matsuo-Takasaki, M., Sato, S. M., and Sargent, T. D. (1999). Inhibitory patterning of the anterior neural plate in Xenopus by homeodomain factors Dlx3 and Msx1. Dev Biol *212*, 455-464.
Feng, X. H., and Derynck, R. (2005). Specificity and versatility in tgf-beta signaling through Smads. Annu Rev Cell Dev Biol *21*, 659-693.
Fletcher, R. B., Baker, J. C., and Harland, R. M. (2006). FGF8 spliceforms mediate early mesoderm and posterior neural tissue formation in Xenopus. Development *133*, 1703-1714.
Fletcher, R. B., and Harland, R. M. (2008). The role of FGF signaling in the establishment and maintenance of mesodermal gene expression in Xenopus. Dev Dyn *237*, 1243-1254.
Fuentealba, L. C., Eivers, E., Ikeda, A., Hurtado, C., Kuroda, H., Pera, E. M., and De Robertis, E. M. (2007). Integrating patterning signals: Wnt/GSK3 regulates the duration of the BMP/Smad1 signal. Cell *131*, 980-993.
Fukuchi, M., Imamura, T., Chiba, T., Ebisawa, T., Kawabata, M., Tanaka, K., and Miyazono, K. (2001). Ligand-dependent degradation of Smad3 by a ubiquitin ligase complex of ROC1 and associated proteins. Mol Biol Cell *12*, 1431-1443.
Funakoshi, Y., Doi, Y., Hosoda, N., Uchida, N., Osawa, M., Shimada, I., Tsujimoto, M., Suzuki, T., Katada, T., and Hoshino, S. (2007). Mechanism of mRNA deadenylation: evidence for a molecular interplay between translation termination factor eRF3 and mRNA deadenylases. Genes Dev *21*, 3135-3148.
Gawantka, V., Delius, H., Hirschfeld, K., Blumenstock, C., and Niehrs, C. (1995). Antagonizing the Spemann organizer: role of the homeobox gene Xvent-1. Embo J *14*, 6268-6279.
Ge, G., and Greenspan, D. S. (2006). Developmental roles of the BMP1/TLD metalloproteinases. Birth Defects Res C Embryo Today *78*, 47-68.

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*, 357-362.
Godsave, S. F., and Slack, J. M. (1989). Clonal analysis of mesoderm induction in Xenopus laevis. Dev Biol *134*, 486-490.
Goto, K., Kamiya, Y., Imamura, T., Miyazono, K., and Miyazawa, K. (2007). Selective inhibitory effects of Smad6 on bone morphogenetic protein type I receptors. J Biol Chem *282*, 20603-20611.
Graff, J. M. (1997). Embryonic patterning: to BMP or not to BMP, that is the question. Cell *89*, 171-174.
Gray, A. M., and Mason, A. J. (1990). Requirement for activin A and transforming growth factor--beta 1 pro-regions in homodimer assembly. Science *247*, 1328-1330.
Grimm, O. H., and Gurdon, J. B. (2002). Nuclear exclusion of Smad2 is a mechanism leading to loss of competence. Nat Cell Biol *4*, 519-522.
Gritsman, K., Zhang, J., Cheng, S., Heckscher, E., Talbot, W. S., and Schier, A. F. (1999). The EGF-CFC protein one-eyed pinhead is essential for nodal signaling. Cell *97*, 121-132.
Gronroos, E., Hellman, U., Heldin, C. H., and Ericsson, J. (2002). Control of Smad7 stability by competition between acetylation and ubiquitination. Mol Cell *10*, 483-493.
Gross, S. R., and Kinzy, T. G. (2005). Translation elongation factor 1A is essential for regulation of the actin cytoskeleton and cell morphology. Nat Struct Mol Biol *12*, 772-778.
Guo, X., Ramirez, A., Waddell, D. S., Li, Z., Liu, X., and Wang, X. F. (2008). Axin and GSK3- control Smad3 protein stability and modulate TGF- signaling. Genes Dev *22*, 106-120.
Gurdon, J. B., Lane, C. D., Woodland, H. R., and Marbaix, G. (1971). Use of frog eggs and oocytes for the study of messenger RNA and its translation in living cells. Nature *233*, 177-182.
Hammerschmidt, M., and Mullins, M. C. (2002). Dorsoventral patterning in the zebrafish: bone morphogenetic proteins and beyond. Results Probl Cell Differ *40*, 72-95.
Hansen, C. S., Marion, C. D., Steele, K., George, S., and Smith, W. C. (1997). Direct neural induction and selective inhibition of mesoderm and epidermis inducers by Xnr3. Development *124*, 483-492.
Haramoto, Y., Tanegashima, K., Onuma, Y., Takahashi, S., Sekizaki, H., and Asashima, M. (2004). Xenopus tropicalis nodal-related gene 3 regulates BMP signaling: an essential role for the pro-region. Dev Biol *265*, 155-168.
Harland, R. (2000). Neural induction. Curr Opin Genet Dev *10*, 357-362.
Harland, R., and Gerhart, J. (1997). Formation and function of Spemann's organizer. Annu Rev Cell Dev Biol *13*, 611-667.
Harrington, A. E., Morris-Triggs, S. A., Ruotolo, B. T., Robinson, C. V., Ohnuma, S., and Hyvonen, M. (2006). Structural basis for the inhibition of activin signalling by follistatin. Embo J *25*, 1035-1045.
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*, 186-197.
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*, 186-197.
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*, 229-240.
Hayashi, H., Abdollah, S., Qiu, Y., Cai, J., Xu, Y. Y., Grinnell, B. W., Richardson, M. A., Topper, J. N., Gimbrone, M. A., Jr., Wrana, J. L., and Falb, D. (1997). The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signaling. Cell *89*, 1165-1173.
Hayes, S., Chawla, A., and Corvera, S. (2002). TGF beta receptor internalization into EEA1-enriched early endosomes: role in signaling to Smad2. J Cell Biol *158*, 1239-1249.
Heasman, J. (2006). Patterning the early Xenopus embryo. Development *133*, 1205-1217.
Heeg-Truesdell, E., and LaBonne, C. (2006). Neural induction in Xenopus requires inhibition of Wnt-beta-catenin signaling. Dev Biol *298*, 71-86.
Heldin, C. H., Miyazono, K., and ten Dijke, P. (1997). TGF-beta signalling from cell membrane to nucleus through SMAD proteins. Nature *390*, 465-471.
Hemmati-Brivanlou, A., and Melton, D. (1997). Vertebrate neural induction. Annu Rev Neurosci *20*, 43-60.
Henningfeld, K. A., Friedle, H., Rastegar, S., and Knochel, W. (2002). Autoregulation of Xvent-2B; direct interaction and functional cooperation of Xvent-2 and Smad1. J Biol Chem *277*, 2097-2103.
Henningfeld, K. A., Rastegar, S., Adler, G., and Knochel, W. (2000). Smad1 and Smad4 are components of the bone morphogenetic protein-4 (BMP-4)-induced transcription complex of the Xvent-2B promoter. J Biol Chem *275*, 21827-21835.
Hill, C. S. (2001). TGF-beta signalling pathways in early Xenopus development. Curr Opin Genet Dev *11*, 533-540.
Hocevar, B. A., Smine, A., Xu, X. X., and Howe, P. H. (2001). The adaptor molecule Disabled-2 links the transforming growth factor beta receptors to the Smad pathway. Embo J *20*, 2789-2801.
Houston, D. W., and Wylie, C. (2005). Maternal Xenopus Zic2 negatively regulates Nodal-related gene expression during anteroposterior patterning. Development *132*, 4845-4855.
Howell, M., Mohun, T. J., and Hill, C. S. (2001). Xenopus Smad3 is specifically expressed in the chordoneural hinge, notochord and in the endocardium of the developing heart. Mech Dev *104*, 147-150.
Iemura, S., Yamamoto, T. S., Takagi, C., Uchiyama, H., Natsume, T., Shimasaki, S., Sugino, H., and Ueno, N. (1998). Direct binding of follistatin to a complex of bone-morphogenetic protein and its receptor inhibits ventral and epidermal cell fates in early Xenopus embryo. Proc Natl Acad Sci U S A *95*,

9337-9342.
Imai, Y., Gates, M. A., Melby, A. E., Kimelman, D., Schier, A. F., and Talbot, W. S. (2001). The homeobox genes vox and vent are redundant repressors of dorsal fates in zebrafish. Development *128*, 2407-2420.
Imamura, T., Takase, M., Nishihara, A., Oeda, E., Hanai, J., Kawabata, M., and Miyazono, K. (1997). Smad6 inhibits signalling by the TGF-beta superfamily. Nature *389*, 622-626.
Inagaki, Y., Blouin, C., Susko, E., and Roger, A. J. (2003). Assessing functional divergence in EF-1alpha and its paralogs in eukaryotes and archaebacteria. Nucleic Acids Res *31*, 4227-4237.
Inman, G. J., Nicolas, F. J., and Hill, C. S. (2002). Nucleocytoplasmic shuttling of Smads 2, 3, and 4 permits sensing of TGF-beta receptor activity. Mol Cell *10*, 283-294.
Isaacs, H. V., Deconinck, A. E., and Pownall, M. E. (2007). FGF4 regulates blood and muscle specification in Xenopus laevis. Biol Cell *99*, 165-173.
Ishimura, A., Ng, J. K., Taira, M., Young, S. G., and Osada, S. (2006). Man1, an inner nuclear membrane protein, regulates vascular remodeling by modulating transforming growth factor beta signaling. Development *133*, 3919-3928.
Itoh, S., and ten Dijke, P. (2007). Negative regulation of TGF-beta receptor/Smad signal transduction. Curr Opin Cell Biol *19*, 176-184.
Janknecht, R., Wells, N. J., and Hunter, T. (1998). TGF-beta-stimulated cooperation of smad proteins with the coactivators CBP/p300. Genes Dev *12*, 2114-2119.
Jiao, K., Zhou, Y., and Hogan, B. L. (2002). Identification of mZnf8, a mouse Kruppel-like transcriptional repressor, as a novel nuclear interaction partner of Smad1. Mol Cell Biol *22*, 7633-7644.
J**onas, E. A., Snape, A. M., and Sargent, T. D.** (1989). Transcriptional regulation of a Xenopus embryonic epidermal keratin gene. Development *106*, 399-405.
Jones, C. M., Dale, L., Hogan, B. L., Wright, C. V., and Smith, J. C. (1996). Bone morphogenetic protein-4 (BMP-4) acts during gastrula stages to cause ventralization of Xenopus embryos. Development *122*, 1545-1554.
Kamaraju, A. K., and Roberts, A. B. (2005). Role of Rho/ROCK and p38 MAP kinase pathways in transforming growth factor-beta-mediated Smad-dependent growth inhibition of human breast carcinoma cells in vivo. J Biol Chem *280*, 1024-1036.
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*, 1365-1375.
Kawano, Y., and Kypta, R. (2003). Secreted antagonists of the Wnt signalling pathway. J Cell Sci *116*, 2627-2634.
Khokha, M. K., Yeh, J., Grammer, T. C., and Harland, R. M. (2005). Depletion of three BMP antagonists from Spemann's organizer leads to a catastrophic loss of dorsal structures. Dev Cell *8*, 401-411.
Kimelman, D. (2006). Mesoderm induction: from caps to chips. Nat Rev Genet *7*, 360-372.

Knockaert, M., Sapkota, G., Alarcon, C., Massague, J., and Brivanlou, A. H. (2006). Unique players in the BMP pathway: small C-terminal domain phosphatases dephosphorylate Smad1 to attenuate BMP signaling. Proc Natl Acad Sci U S A *103*, 11940-11945.
Koinuma, D., Shinozaki, M., Komuro, A., Goto, K., Saitoh, M., Hanyu, A., Ebina, M., Nukiwa, T., Miyazawa, K., Imamura, T., and Miyazono, K. (2003). Arkadia amplifies TGF-beta superfamily signalling through degradation of Smad7. Embo J *22*, 6458-6470.
Komuro, A., Imamura, T., Saitoh, M., Yoshida, Y., Yamori, T., Miyazono, K., and Miyazawa, K. (2004). Negative regulation of transforming growth factor-beta (TGF-beta) signaling by WW domain-containing protein 1 (WWP1). Oncogene *23*, 6914-6923.
Krakowski, A. R., Laboureau, J., Mauviel, A., Bissell, M. J., and Luo, K. (2005). Cytoplasmic SnoN in normal tissues and nonmalignant cells antagonizes TGF-beta signaling by sequestration of the Smad proteins. Proc Natl Acad Sci U S A *102*, 12437-12442.
Kretzschmar, M., Doody, J., and Massague, J. (1997). Opposing BMP and EGF signalling pathways converge on the TGF-beta family mediator Smad1. Nature *389*, 618-622.
Kretzschmar, M., Doody, J., Timokhina, I., and Massague, J. (1999). A mechanism of repression of TGFbeta/ Smad signaling by oncogenic Ras. Genes Dev *13*, 804-816.
Ku, M., and Melton, D. A. (1993). Xwnt-11: a maternally expressed Xenopus wnt gene. Development *119*, 1161-1173.
Kudo, H., Senju, S., Mitsuya, H., and Nishimura, Y. (2000). Mouse and human GTPBP2, newly identified members of the GP-1 family of GTPase. Biochem Biophys Res Commun *272*, 456-465.
Kudoh, T., Concha, M. L., Houart, C., Dawid, I. B., and Wilson, S. W. (2004). Combinatorial Fgf and Bmp signalling patterns the gastrula ectoderm into prospective neural and epidermal domains. Development *131*, 3581-3592.
Kume, S., Haneda, M., Kanasaki, K., Sugimoto, T., Araki, S., Isshiki, K., Isono, M., Uzu, T., Guarente, L., Kashiwagi, A., and Koya, D. (2007). SIRT1 inhibits transforming growth factor beta-induced apoptosis in glomerular mesangial cells via Smad7 deacetylation. J Biol Chem *282*, 151-158.
Kuratomi, G., Komuro, A., Goto, K., Shinozaki, M., Miyazawa, K., Miyazono, K., and Imamura, T. (2005). NEDD4-2 (neural precursor cell expressed, developmentally down-regulated 4-2) negatively regulates TGF-beta (transforming growth factor-beta) signalling by inducing ubiquitin-mediated degradation of Smad2 and TGF-beta type I receptor. Biochem J *386*, 461-470.
Kuroda, H., Fuentealba, L., Ikeda, A., Reversade, B., and De Robertis, E. M. (2005). Default neural induction: neuralization of dissociated Xenopus cells is mediated by Ras/MAPK activation. Genes Dev *19*, 1022-1027.
Kuroda, H., Wessely, O., and De Robertis, E. M. (2004). Neural induction in Xenopus: requirement for ectodermal and endomesodermal signals via Chordin, Noggin, beta-Catenin, and Cerberus. PLoS Biol *2*, E92.
LaBonne, C., Burke, B., and Whitman, M. (1995). Role of MAP kinase in

mesoderm induction and axial patterning during Xenopus development. Development *121*, 1475-1486.
Larabell, C. A., Torres, M., Rowning, B. A., Yost, C., Miller, J. R., Wu, M., Kimelman, D., and Moon, R. T. (1997). Establishment of the dorso-ventral axis in Xenopus embryos is presaged by early asymmetries in beta-catenin that are modulated by the Wnt signaling pathway. J Cell Biol *136*, 1123-1136.
Latinkic, B. V., Umbhauer, M., Neal, K. A., Lerchner, W., Smith, J. C., and Cunliffe, V. (1997). The Xenopus Brachyury promoter is activated by FGF and low concentrations of activin and suppressed by high concentrations of activin and by paired-type homeodomain proteins. Genes Dev *11*, 3265-3276.
Lebreton, S., and Jones, C. M. (2006). Emergence of organizer function: a lot of "stuff" involved. Semin Cell Dev Biol *17*, 110-116.
Leclercq, T. M., Moretti, P. A., Vadas, M. A., and Pitson, S. M. (2008). Eukaryotic elongation factor 1A interacts with sphingosine kinase and directly enhances its catalytic activity. J Biol Chem *283*, 9606-9614.
Lee, J., Son, M. J., Woolard, K., Donin, N. M., Li, A., Cheng, C. H., Kotliarova, S., Kotliarov, Y., Walling, J., Ahn, S.*, et al.* (2008). Epigenetic-mediated dysfunction of the bone morphogenetic protein pathway inhibits differentiation of glioblastoma-initiating cells. Cancer Cell *13*, 69-80.
Lee, J. A., Park, J. E., Lee, D. H., Park, S. G., Myung, P. K., Park, B. C., and Cho, S. (2008). G1 to S phase transition protein 1 induces apoptosis signal-regulating kinase 1 activation by dissociating 14-3-3 from ASK1. Oncogene *27*, 1297-1305.
Lee, P. S., Chang, C., Liu, D., and Derynck, R. (2003). Sumoylation of Smad4, the common Smad mediator of transforming growth factor-beta family signaling. J Biol Chem *278*, 27853-27863.
Lemaire, P., Bertrand, V., and Hudson, C. (2002). Early steps in the formation of neural tissue in ascidian embryos. Dev Biol *252*, 151-169.
Lin, H. K., Bergmann, S., and Pandolfi, P. P. (2004). Cytoplasmic PML function in TGF-beta signalling. Nature *431*, 205-211.
Lin, X., Duan, X., Liang, Y. Y., Su, Y., Wrighton, K. H., Long, J., Hu, M., Davis, C. M., Wang, J., Brunicardi, F. C.*, et al.* (2006). PPM1A functions as a Smad phosphatase to terminate TGFbeta signaling. Cell *125*, 915-928.
Lin, X., Liang, M., and Feng, X. H. (2000). Smurf2 is a ubiquitin E3 ligase mediating proteasome-dependent degradation of Smad2 in transforming growth factor-beta signaling. J Biol Chem *275*, 36818-36822.
Lin, X., Liang, M., Liang, Y. Y., Brunicardi, F. C., Melchior, F., and Feng, X. H. (2003). Activation of transforming growth factor-beta signaling by SUMO-1 modification of tumor suppressor Smad4/DPC4. J Biol Chem *278*, 18714-18719.
Lin, X., Liang, Y. Y., Sun, B., Liang, M., Shi, Y., Brunicardi, F. C., Shi, Y., and Feng, X. H. (2003). Smad6 recruits transcription corepressor CtBP to repress bone morphogenetic protein-induced transcription. Mol Cell Biol *23*, 9081-9093.
Linker, C., and Stern, C. D. (2004). Neural induction requires BMP inhibition only as a late step, and involves signals other than FGF and Wnt antagonists. Development *131*, 5671-5681.
Lo, R. S., and Massague, J. (1999). Ubiquitin-dependent degradation of TGF-

beta-activated smad2. Nat Cell Biol *1*, 472-478.
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*, 785-795.
Luo, K., and Lodish, H. F. (1996). Signaling by chimeric erythropoietin-TGF-beta receptors: homodimerization of the cytoplasmic domain of the type I TGF-beta receptor and heterodimerization with the type II receptor are both required for intracellular signal transduction. Embo J *15*, 4485-4496.
Luo, K., Stroschein, S. L., Wang, W., Chen, D., Martens, E., Zhou, S., and Zhou, Q. (1999). The Ski oncoprotein interacts with the Smad proteins to repress TGFbeta signaling. Genes Dev *13*, 2196-2206.
Luo, T., Matsuo-Takasaki, M., Lim, J. H., and Sargent, T. D. (2001). Differential regulation of Dlx gene expression by a BMP morphogenetic gradient. Int J Dev Biol *45*, 681-684.
Luo, T., Matsuo-Takasaki, M., Thomas, M. L., Weeks, D. L., and Sargent, T. D. (2002). Transcription factor AP-2 is an essential and direct regulator of epidermal development in Xenopus. Dev Biol *245*, 136-144.
Marom, K., Fainsod, A., and Steinbeisser, H. (1999). Patterning of the mesoderm involves several threshold responses to BMP-4 and Xwnt-8. Mech Dev *87*, 33-44.
Marom, K., Levy, V., Pillemer, G., and Fainsod, A. (2005). Temporal analysis of the early BMP functions identifies distinct anti-organizer and mesoderm patterning phases. Dev Biol *282*, 442-454.
Massague, J., Seoane, J., and Wotton, D. (2005). Smad transcription factors. Genes Dev *19*, 2783-2810.
Massague, J., and Wotton, D. (2000). Transcriptional control by the TGF-beta/Smad signaling system. Embo J *19*, 1745-1754.
Masse, K., Baldwin, R., Barnett, M. W., and Jones, E. A. (2004). X-epilectin: a novel epidermal fucolectin regulated by BMP signalling. Int J Dev Biol *48*, 1119-1129.
Matsuura, I., Denissova, N. G., Wang, G., He, D., Long, J., and Liu, F. (2004). Cyclin-dependent kinases regulate the antiproliferative function of Smads. Nature *430*, 226-231.
Mauxion, F., Faux, C., and Seraphin, B. (2008). The BTG2 protein is a general activator of mRNA deadenylation. Embo J.
Melby, A. E., Beach, C., Mullins, M., and Kimelman, D. (2000). Patterning the early zebrafish by the opposing actions of bozozok and vox/vent. Dev Biol *224*, 275-285.
Meno, C., Shimono, A., Saijoh, Y., Yashiro, K., Mochida, K., Ohishi, S., Noji, S., Kondoh, H., and Hamada, H. (1998). lefty-1 is required for left-right determination as a regulator of lefty-2 and nodal. Cell *94*, 287-297.
Miura, S., Takeshita, T., Asao, H., Kimura, Y., Murata, K., Sasaki, Y., Hanai, J. I., Beppu, H., Tsukazaki, T., Wrana, J. L.*, et al.* (2000). Hgs (Hrs), a FYVE domain protein, is involved in Smad signaling through cooperation with SARA. Mol Cell Biol *20*, 9346-9355.
Miyasaka, T., Morita, M., Ito, K., Suzuki, T., Fukuda, H., Takeda, S., Inoue, J.,

Semba, K., and Yamamoto, T. (2008). Interaction of antiproliferative protein Tob with the CCR4-NOT deadenylase complex. Cancer Sci *99*, 755-761.
Moos, M., Jr., Wang, S., and Krinks, M. (1995). Anti-dorsalizing morphogenetic protein is a novel TGF-beta homolog expressed in the Spemann organizer. Development *121*, 4293-4301.
Moreno, M., Munoz, R., Aroca, F., Labarca, M., Brandan, E., and Larrain, J. (2005). Biglycan is a new extracellular component of the Chordin-BMP4 signaling pathway. Embo J *24*, 1397-1405.
Mori, S., Matsuzaki, K., Yoshida, K., Furukawa, F., Tahashi, Y., Yamagata, H., Sekimoto, G., Seki, T., Matsui, H., Nishizawa, M.*, et al.* (2004). TGF-beta and HGF transmit the signals through JNK-dependent Smad2/3 phosphorylation at the linker regions. Oncogene *23*, 7416-7429.
Mulholland, P. J., Fiegler, H., Mazzanti, C., Gorman, P., Sasieni, P., Adams, J., Jones, T. A., Babbage, J. W., Vatcheva, R., Ichimura, K.*, et al.* (2006). Genomic profiling identifies discrete deletions associated with translocations in glioblastoma multiforme. Cell Cycle *5*, 783-791.
Munoz-Sanjuan, I., and Brivanlou, A. H. (2002). Neural induction, the default model and embryonic stem cells. Nat Rev Neurosci *3*, 271-280.
Oelgeschlager, M., Larrain, J., Geissert, D., and De Robertis, E. M. (2000). The evolutionarily conserved BMP-binding protein Twisted gastrulation promotes BMP signalling. Nature *405*, 757-763.
Onichtchouk, D., Chen, Y. G., Dosch, R., Gawantka, V., Delius, H., Massague, J., and Niehrs, C. (1999). Silencing of TGF-beta signalling by the pseudoreceptor BAMBI. Nature *401*, 480-485.
Onichtchouk, D., Gawantka, V., Dosch, R., Delius, H., Hirschfeld, K., Blumenstock, C., and Niehrs, C. (1996). The Xvent-2 homeobox gene is part of the BMP-4 signalling pathway controlling [correction of controling] dorsoventral patterning of Xenopus mesoderm. Development *122*, 3045-3053.
Osada, S., Ohmori, S. Y., and Taira, M. (2003). XMAN1, an inner nuclear membrane protein, antagonizes BMP signaling by interacting with Smad1 in Xenopus embryos. Development *130*, 1783-1794.
Pera, E. M., and De Robertis, E. M. (2000). A direct screen for secreted proteins in Xenopus embryos identifies distinct activities for the Wnt antagonists Crescent and Frzb-1. Mech Dev *96*, 183-195.
Piccirillo, S. G., Reynolds, B. A., Zanetti, N., Lamorte, G., Binda, E., Broggi, G., Brem, H., Olivi, A., Dimeco, F., and Vescovi, A. L. (2006). Bone morphogenetic proteins inhibit the tumorigenic potential of human brain tumour-initiating cells. Nature *444*, 761-765.
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*, 707-710.
Piccolo, S., Agius, E., Lu, B., Goodman, S., Dale, L., and De Robertis, E. M. (1997). Cleavage of Chordin by Xolloid metalloprotease suggests a role for proteolytic processing in the regulation of Spemann organizer activity. Cell *91*, 407-416.
Piccolo, S., Sasai, Y., Lu, B., and De Robertis, E. M. (1996). Dorsoventral

patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP-4. Cell *86*, 589-598.
Piepenburg, O., Grimmer, D., Williams, P. H., and Smith, J. C. (2004). Activin redux: specification of mesodermal pattern in Xenopus by graded concentrations of endogenous activin B. Development *131*, 4977-4986.
Polack, S. S. (1949). The xenopus pregnancy test. Can Med Assoc J *60*, 159-161.
Raju, G. P., Dimova, N., Klein, P. S., and Huang, H. C. (2003). SANE, a novel LEM domain protein, regulates bone morphogenetic protein signaling through interaction with Smad1. J Biol Chem *278*, 428-437.
Rentzsch, F., Bakkers, J., Kramer, C., and Hammerschmidt, M. (2004). Fgf signaling induces posterior neuroectoderm independently of Bmp signaling inhibition. Dev Dyn *231*, 750-757.
Reversade, B., Kuroda, H., Lee, H., Mays, A., and De Robertis, E. M. (2005). Depletion of Bmp2, Bmp4, Bmp7 and Spemann organizer signals induces massive brain formation in Xenopus embryos. Development *132*, 3381-3392.
Rex, M., Hilton, E., and Old, R. (2002). Multiple interactions between maternally-activated signalling pathways control Xenopus nodal-related genes. Int J Dev Biol *46*, 217-226.
Rho, S. B., Park, Y. G., Park, K., Lee, S. H., and Lee, J. H. (2006). A novel cervical cancer suppressor 3 (CCS-3) interacts with the BTB domain of PLZF and inhibits the cell growth by inducing apoptosis. FEBS Lett *580*, 4073-4080.
Ribisi, S., Jr., Mariani, F. V., Aamar, E., Lamb, T. M., Frank, D., and Harland, R. M. (2000). Ras-mediated FGF signaling is required for the formation of posterior but not anterior neural tissue in Xenopus laevis. Dev Biol *227*, 183-196.
Ross, S., Cheung, E., Petrakis, T. G., Howell, M., Kraus, W. L., and Hill, C. S. (2006). Smads orchestrate specific histone modifications and chromatin remodeling to activate transcription. Embo J *25*, 4490-4502.
Ross, S., and Hill, C. S. (2008). How the Smads regulate transcription. Int J Biochem Cell Biol *40*, 383-408.
Sammar, M., Stricker, S., Schwabe, G. C., Sieber, C., Hartung, A., Hanke, M., Oishi, I., Pohl, J., Minami, Y., Sebald, W.*, et al.* (2004). Modulation of GDF5/BRI-b signalling through interaction with the tyrosine kinase receptor Ror2. Genes Cells *9*, 1227-1238.
Sander, V., Reversade, B., and De Robertis, E. M. (2007). The opposing homeobox genes Goosecoid and Vent1/2 self-regulate Xenopus patterning. Embo J *26*, 2955-2965.
Sapkota, G., Alarcon, C., Spagnoli, F. M., Brivanlou, A. H., and Massague, J. (2007). Balancing BMP signaling through integrated inputs into the Smad1 linker. Mol Cell *25*, 441-454.
Sapkota, G., Knockaert, M., Alarcon, C., Montalvo, E., Brivanlou, A. H., and Massague, J. (2006). Dephosphorylation of the linker regions of Smad1 and Smad2/3 by small C-terminal domain phosphatases has distinct outcomes for bone morphogenetic protein and transforming growth factor-beta pathways. J Biol Chem *281*, 40412-40419.
Sasai, Y., and De Robertis, E. M. (1997). Ectodermal patterning in vertebrate

embryos. Dev Biol *182*, 5-20.
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*, 779-790.
Sasaki, A., Masuda, Y., Ohta, Y., Ikeda, K., and Watanabe, K. (2001). Filamin associates with Smads and regulates transforming growth factor-beta signaling. J Biol Chem *276*, 17871-17877.
Sato, S. M., and Sargent, T. D. (1991). Localized and inducible expression of Xenopus-posterior (Xpo), a novel gene active in early frog embryos, encoding a protein with a 'CCHC' finger domain. Development *112*, 747-753.
Scherner, O., Meurer, S. K., Tihaa, L., Gressner, A. M., and Weiskirchen, R. (2007). Endoglin differentially modulates antagonistic transforming growth factor-beta1 and BMP-7 signaling. J Biol Chem *282*, 13934-13943.
Schier, A. F., and Talbot, W. S. (2005). Molecular genetics of axis formation in zebrafish. Annu Rev Genet *39*, 561-613.
Schmerer, M., and Evans, T. (2003). Primitive erythropoiesis is regulated by Smad-dependent signaling in postgastrulation mesoderm. Blood *102*, 3196-3205.
Schmierer, B., and Hill, C. S. (2005). Kinetic analysis of Smad nucleocytoplasmic shuttling reveals a mechanism for transforming growth factor beta-dependent nuclear accumulation of Smads. Mol Cell Biol *25*, 9845-9858.
Schmierer, B., and Hill, C. S. (2007). TGFbeta-SMAD signal transduction: molecular specificity and functional flexibility. Nat Rev Mol Cell Biol *8*, 970-982.
Schneider, V. A., and Mercola, M. (2001). Wnt antagonism initiates cardiogenesis in Xenopus laevis. Genes Dev *15*, 304-315.
Schohl, A., and Fagotto, F. (2002). Beta-catenin, MAPK and Smad signaling during early Xenopus development. Development *129*, 37-52.
Senju, S., Iyama, K., Kudo, H., Aizawa, S., and Nishimura, Y. (2000). Immunocytochemical analyses and targeted gene disruption of GTPBP1. Mol Cell Biol *20*, 6195-6200.
Senju, S., and Nishimura, Y. (1997). Identification of human and mouse GP-1, a putative member of a novel G-protein family. Biochem Biophys Res Commun *231*, 360-364.
Seoane, J., Le, H. V., Shen, L., Anderson, S. A., and Massague, J. (2004). Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. Cell *117*, 211-223.
Shamovsky, I., Ivannikov, M., Kandel, E. S., Gershon, D., and Nudler, E. (2006). RNA-mediated response to heat shock in mammalian cells. Nature *440*, 556-560.
Shi, W., Chang, C., Nie, S., Xie, S., Wan, M., and Cao, X. (2007). Endofin acts as a Smad anchor for receptor activation in BMP signaling. J Cell Sci *120*, 1216-1224.
Shi, W., Sun, C., He, B., Xiong, W., Shi, X., Yao, D., and Cao, X. (2004). GADD34-PP1c recruited by Smad7 dephosphorylates TGFbeta type I receptor. J Cell Biol *164*, 291-300.
Shi, Y., and Massague, J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell *113*, 685-700.

Shi, Y., Wang, Y. F., Jayaraman, L., Yang, H., Massague, J., and Pavletich, N. P. (1998). Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGF-beta signaling. Cell *94*, 585-594.
Shibata, M., Itoh, M., Hikasa, H., Taira, S., and Taira, M. (2005). Role of crescent in convergent extension movements by modulating Wnt signaling in early Xenopus embryogenesis. Mech Dev *122*, 1322-1339.
Silva, A. C., Filipe, M., Kuerner, K. M., Steinbeisser, H., and Belo, J. A. (2003). Endogenous Cerberus activity is required for anterior head specification in Xenopus. Development *130*, 4943-4953.
Simeoni, I., and Gurdon, J. B. (2007). Interpretation of BMP signaling in early Xenopus development. Dev Biol *308*, 82-92.
Simonsson, M., Heldin, C. H., Ericsson, J., and Gronroos, E. (2005). The balance between acetylation and deacetylation controls Smad7 stability. J Biol Chem *280*, 21797-21803.
Song, J., Durrin, L. K., Wilkinson, T. A., Krontiris, T. G., and Chen, Y. (2004). Identification of a SUMO-binding motif that recognizes SUMO-modified proteins. Proc Natl Acad Sci U S A *101*, 14373-14378.
Spemann, H., and Mangold, H. (2001). Induction of embryonic primordia by implantation of organizers from a different species. 1923. Int J Dev Biol *45*, 13-38.
Sprang, S. R. (1997). G protein mechanisms: insights from structural analysis. Annu Rev Biochem *66*, 639-678.
Stern, C. D. (2002). Induction and initial patterning of the nervous system - the chick embryo enters the scene. Curr Opin Genet Dev *12*, 447-451.
Stern, C. D. (2005). Neural induction: old problem, new findings, yet more questions. Development *132*, 2007-2021.
Sudarwati, S. a. P. D. N. (1971). Mesoderm formation in the anuran Xenopus laevis (Daudin). . W Roux' Arch 189-204
Suzuki, A., Kaneko, E., Ueno, N., and Hemmati-Brivanlou, A. (1997). Regulation of epidermal induction by BMP2 and BMP7 signaling. Dev Biol *189*, 112-122.
Suzuki, A., Ueno, N., and Hemmati-Brivanlou, A. (1997). Xenopus msx1 mediates epidermal induction and neural inhibition by BMP4. Development *124*, 3037-3044.
Szeto, D. P., and Kimelman, D. (2004). Combinatorial gene regulation by Bmp and Wnt in zebrafish posterior mesoderm formation. Development *131*, 3751-3760.
Takahashi, S., Yokota, C., Takano, K., Tanegashima, K., Onuma, Y., Goto, J., and Asashima, M. (2000). Two novel nodal-related genes initiate early inductive events in Xenopus Nieuwkoop center. Development *127*, 5319-5329.
Takeda, M., Saito, Y., Sekine, R., Onitsuka, I., Maeda, R., and Maeno, M. (2000). Xenopus msx-1 regulates dorso-ventral axis formation by suppressing the expression of organizer genes. Comp Biochem Physiol B Biochem Mol Biol *126*, 157-168.
Tao, J., Kuliyev, E., Wang, X., Li, X., Wilanowski, T., Jane, S. M., Mead, P. E., and Cunningham, J. M. (2005). BMP4-dependent expression of Xenopus Grainyhead-like 1 is essential for epidermal differentiation. Development *132*,

1021-1034.
Taverner, N. V., Kofron, M., Shin, Y., Kabitschke, C., Gilchrist, M. J., Wylie, C., Cho, K. W., Heasman, J., and Smith, J. C. (2005). Microarray-based identification of VegT targets in Xenopus. Mech Dev *122*, 333-354.
Ten Dijke, P., Goumans, M. J., Itoh, F., and Itoh, S. (2002). Regulation of cell proliferation by Smad proteins. J Cell Physiol *191*, 1-16.
ten Dijke, P., and Hill, C. S. (2004). New insights into TGF-beta-Smad signalling. Trends Biochem Sci *29*, 265-273.
Thomsen, G. H. (1996). Xenopus mothers against decapentaplegic is an embryonic ventralizing agent that acts downstream of the BMP-2/4 receptor. Development *122*, 2359-2366.
Tsukazaki, T., Chiang, T. A., Davison, A. F., Attisano, L., and Wrana, J. L. (1998). SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. Cell *95*, 779-791.
Vetter, I. R., and Wittinghofer, A. (2001). The guanine nucleotide-binding switch in three dimensions. Science *294*, 1299-1304.
Vonica, A., and Gumbiner, B. M. (2002). Zygotic Wnt activity is required for Brachyury expression in the early Xenopus laevis embryo. Dev Biol *250*, 112-127.
Wagner, T. U. (2007). Bone morphogenetic protein signaling in stem cells--one signal, many consequences. Febs J *274*, 2968-2976.
Wan, M., Tang, Y., Tytler, E. M., Lu, C., Jin, B., Vickers, S. M., Yang, L., Shi, X., and Cao, X. (2004). Smad4 protein stability is regulated by ubiquitin ligase SCF beta-TrCP1. J Biol Chem *279*, 14484-14487.
Wardle, F. C., and Smith, J. C. (2006). Transcriptional regulation of mesendoderm formation in Xenopus. Semin Cell Dev Biol *17*, 99-109.
Wardle, F. C., Welch, J. V., and Dale, L. (1999). Bone morphogenetic protein 1 regulates dorsal-ventral patterning in early Xenopus embryos by degrading chordin, a BMP4 antagonist. Mech Dev *86*, 75-85.
Warner, D. R., Pisano, M. M., Roberts, E. A., and Greene, R. M. (2003). Identification of three novel Smad binding proteins involved in cell polarity. FEBS Lett *539*, 167-173.
Warner, D. R., Roberts, E. A., Greene, R. M., and Pisano, M. M. (2003). Identification of novel Smad binding proteins. Biochem Biophys Res Commun *312*, 1185-1190.
Watabe, T., Kim, S., Candia, A., Rothbacher, U., Hashimoto, C., Inoue, K., and Cho, K. W. (1995). Molecular mechanisms of Spemann's organizer formation: conserved growth factor synergy between Xenopus and mouse. Genes Dev *9*, 3038-3050.
Watanabe, M., Yoshida, K., Hida, M., Kato, H., Uchida, K., Yamaguchi, R., Tateyama, S., and Sugano, S. (2000). Cloning, expression analysis, and chromosomal mapping of GTPBP2, a novel member of the G protein family. Gene *256*, 51-58.
Weaver, C., and Kimelman, D. (2004). Move it or lose it: axis specification in Xenopus. Development *131*, 3491-3499.
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*, 861-867.
Wessely, O., Agius, E., Oelgeschlager, M., Pera, E. M., and De Robertis, E. M. (2001). Neural induction in the absence of mesoderm: beta-catenin-dependent expression of secreted BMP antagonists at the blastula stage in Xenopus. Dev Biol *234*, 161-173.
White, R. J., Sun, B. I., Sive, H. L., and Smith, J. C. (2002). Direct and indirect regulation of derriere, a Xenopus mesoderm-inducing factor, by VegT. Development *129*, 4867-4876.
Wilson, P. A., and Hemmati-Brivanlou, A. (1995). Induction of epidermis and inhibition of neural fate by Bmp-4. Nature *376*, 331-333.
Wilson, P. A., Lagna, G., Suzuki, A., and Hemmati-Brivanlou, A. (1997). Concentration-dependent patterning of the Xenopus ectoderm by BMP4 and its signal transducer Smad1. Development *124*, 3177-3184.
Wilson, S. I., and Edlund, T. (2001). Neural induction: toward a unifying mechanism. Nat Neurosci *4 Suppl*, 1161-1168.
Wilson, S. I., Rydstrom, A., Trimborn, T., Willert, K., Nusse, R., Jessell, T. M., and Edlund, T. (2001). The status of Wnt signalling regulates neural and epidermal fates in the chick embryo. Nature *411*, 325-330.
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*, 1277-1289.
Xanthos, J. B., Kofron, M., Wylie, C., and Heasman, J. (2001). Maternal VegT is the initiator of a molecular network specifying endoderm in Xenopus laevis. Development *128*, 167-180.
Xiong, B., Rui, Y., Zhang, M., Shi, K., Jia, S., Tian, T., Yin, K., Huang, H., Lin, S., Zhao, X.*, et al.* (2006). Tob1 controls dorsal development of zebrafish embryos by antagonizing maternal beta-catenin transcriptional activity. Dev Cell *11*, 225-238.
Xu, L., Kang, Y., Col, S., and Massague, J. (2002). Smad2 nucleocytoplasmic shuttling by nucleoporins CAN/Nup214 and Nup153 feeds TGFbeta signaling complexes in the cytoplasm and nucleus. Mol Cell *10*, 271-282.
Xu, R. H., Ault, K. T., Kim, J., Park, M. J., Hwang, Y. S., Peng, Y., Sredni, D., and Kung, H. (1999). Opposite effects of FGF and BMP-4 on embryonic blood formation: roles of PV.1 and GATA-2. Dev Biol *208*, 352-361.
Yamashita, H., ten Dijke, P., Franzen, P., Miyazono, K., and Heldin, C. H. (1994). Formation of hetero-oligomeric complexes of type I and type II receptors for transforming growth factor-beta. J Biol Chem *269*, 20172-20178.
Yang, J., Tan, C., Darken, R. S., Wilson, P. A., and Klein, P. S. (2002). Beta-catenin/Tcf-regulated transcription prior to the midblastula transition. Development *129*, 5743-5752.
Yeo, C., and Whitman, M. (2001). Nodal signals to Smads through Cripto-dependent and Cripto-independent mechanisms. Mol Cell *7*, 949-957.
Ying, Q. L., Nichols, J., Chambers, I., and Smith, A. (2003). BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell *115*, 281-292.

Yoshida, Y., Tanaka, S., Umemori, H., Minowa, O., Usui, M., Ikematsu, N., Hosoda, E., Imamura, T., Kuno, J., Yamashita, T.*, et al.* (2000). Negative regulation of BMP/Smad signaling by Tob in osteoblasts. Cell *103*, 1085-1097.
Zaidi, S. K., Sullivan, A. J., van Wijnen, A. J., Stein, J. L., Stein, G. S., and Lian, J. B. (2002). Integration of Runx and Smad regulatory signals at transcriptionally active subnuclear sites. Proc Natl Acad Sci U S A *99*, 8048-8053.
Zhang, J., Houston, D. W., King, M. L., Payne, C., Wylie, C., and Heasman, J. (1998). The role of maternal VegT in establishing the primary germ layers in Xenopus embryos. Cell *94*, 515-524.
Zhang, J., and King, M. L. (1996). Xenopus VegT RNA is localized to the vegetal cortex during oogenesis and encodes a novel T-box transcription factor involved in mesodermal patterning. Development *122*, 4119-4129.
Zhu, H., Kavsak, P., Abdollah, S., Wrana, J. L., and Thomsen, G. H. (1999). A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation. Nature *400*, 687-693.
Zimmerman, L. B., De Jesus-Escobar, J. M., and Harland, R. M. (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. Cell *86*, 599-606.

Appendix: List of Clones

Clone	Gene	Vector	Tag-Notes	Clon. Sites	Other
1	GTPBP2B-EST	pCS2G	original EST clone	XhoI-BamHI	shorter form alternatively spliced in human
2	GTPBP2A-EST	pBSRN3	original EST clone	NotI-EcoRI	conserved among all species
3	GTPBP1-EST	pCMV-SPORT6	original EST clone	NotI-SalI	
4	HA-GTPBP2B	pCS2-HA	3xHA	XhoI-XbaI	
5	HA-GTPBP2A	pCS2-HA	3xHA	XhoI-XbaI	
6	HA-GTPBP2-XIs	pCS2-HA	3xHA	XhoI-XbaI	start from a xenopus specific ATG
7	HA-GTPBP2 delN	pCS2-HA	3xHA	XhoI-XbaI	missing GTPase domain
9	HA-GTPBP2B delC	pCS2-HA	3xHA	XhoI-XbaI	GTPase domain only
10	HA-GTPBP2B S17N	pCS2-HA	3xHA	XhoI-XbaI	dominant negative GTPase mutant
11	HA-GTPBP2A S17N	pCS2-HA	3xHA	XhoI-XbaI	dominant negative GTPase mutant
12	HA-GTPBP2B T35Q	pCS2-HA	3xHA	XhoI-XbaI	dominant negative GTPase mutant
13	HA-GTPBP2A T35Q	pCS2-HA	3xHA	XhoI-XbaI	dominant negative GTPase mutant
14	HA-GTPBP2B D80N	pCS2-HA	3xHA	XhoI-XbaI	dominant negative GTPase mutant
15	HA-GTPBP2A D80N	pCS2-HA	3xHA	XhoI-XbaI	dominant negative GTPase mutant
16	HA-GTPBP2B D156N	pCS2-HA	3xHA	XhoI-XbaI	activated GTPAse mutant
17	HA-GTPBP2A D156N	pCS2-HA	3xHA	XhoI-XbaI	activated GTPAse mutant
18	HA-GTPBP2B L19P	pCS2-HA	3xHA	XhoI-XbaI	activated GTPAse mutant
19	HA-GTPBP2A L19P	pCS2-HA	3xHA	XhoI-XbaI	activated GTPAse mutant
20	HA-GTPBP1	pCS2-HA	3xHA	XhoI-XbaI	
21	HA-GTPBP2-XIs-5misx2	pCS2-HA	3xHA	XhoI-XbaI	rescue construct has mismatches against morpholinos M1 and M2
22	HA-GTPBP2A-5mis	pCS2-HA	3xHA	XhoI-XbaI	rescue construct mismatches against M1
23	5'UTR-GTPBP2A-myc	pCS2-myc	1xmyc C terminal	ClaI-NcoI	construct to test morpholino effectiveness
24	HA-GTPBP2B S17A	pCS2-HA	3xHA	XhoI-XbaI	dominant negative GTPase mutant
25	HA-GTPBP2B S17N-T35Q	pCS2-HA	3xHA	XhoI-XbaI	dominant negative GTPase mutant
26	GTPBP2B	pCDNA3.1	no tag	XhoI-XbaI	contsruct to make RNA with T7
27	GTPBP2A	pCDNA3.1	no tag	XhoI-XbaI	contsruct to make RNA with T7
29	GTPBP2B	pCS2	no tag	XhoI-XbaI	contsruct to make RNA with SP6
30	GTPBP2A	pCS2	no tag	XhoI-XbaI	contsruct to make RNA with SP6
31	Sf3b2	pCS2-6myc	6-myc	XhoI-XbaI	
32	pT7.3xHA	pCDNA3.1	3xHA	HindIII-XbaI	vector has HindIII-XbaI casette of pCS2HA. and downstream NotI site for linearization
33	T73xHA-GTPBP2B	pT7.3xHA	3HA	XhoI-XbaI	to make RNA with T7
34	T73xHA-GTPBP2A	pT7.3xHA	3HA	XhoI-XbaI	to make RNA with T7
35	T73xHA-GTPBP2XI-2xmis	pT7.3xHA	3HA	XhoI-XbaI	to make RNA with T7+ morpholino rescue

36	pT7.CS2	pCDNA3.1	no tag	HindIII-XbaI	vector has HindIII-XbaI casette of pCS2.
					and downstream NotI site for linearization
37	pT7.CS2-5mis-GTPBP2A	pT7.CS2	no tag	XhoI-XbaI	untagged rescue contruct
38	pT7.3xHA-Cherry	pT7.3xHA	3xHA + Cherry	XhoI-XhoI	constructed as N' Cherry tag
39	pT7.3xHA-Cherry-GTPBP2B	pT7.3xHA	3xHA + Cherry	XhoI-XbaI	
40	pT7.3xHA-Cherry-GTPBP2A	pT7.3xHA	3xHA + Cherry	XhoI-XbaI	
41	pT7.3xHA-Cherry-GTPBP2A*	pT7.3xHA	3xHA + Cherry	XhoI-XbaI	5mismatch for rescue
42	T73xHA-GTPBP2A S17N	pT7.3xHA	3HA	XhoI-XbaI	dominant negative GTPase mutant
43	T73xHA-GTPBP2A T35Q	pT7.3xHA	3HA	XhoI-XbaI	dominant negative GTPase mutant
44	T73xHA-GTPBP2B T35Q	pT7.3xHA	3HA	XhoI-XbaI	dominant negative GTPase mutant
45	GST-GTPBP2A	pGEX2-2T	GST	EcoRi-XhoI	for protein purification
46	GST-GTPBP2B	pGEX2-2T	GST	EcoRi-XhoI	and antibody production
47	GST-GTPBP2BdelC	pGEX2-2T	GST	EcoRi-XhoI	*
49	GST-GTPBP2delN	pGEX2-2T	GST	EcoRi-XhoI	also invitro binding assays
50	CBD-GTPBP2A	pTYB2	CBD	NdeI-XhoI	for protein purification+Ab
51	GTPBP2A-EST-EGFP	pBSRN3	EGFP	NcoI-NotI	construct to test morpholino effectiveness
52	GTPBP2XI-flag	pCS2	C' flag	XhoI-XbaI	
53	GTPBP2A-flag	pCS2	C' flag	XhoI-XbaI	
54	hGTPBP2A	pCS2-HA	3HA	XhoI-XbaI	human GTPBP2A
55	hGTPBP2B	pCS2-HA	3HA	XhoI-XbaI	human GTPBP2B
56	C'flag-xMad1	pCS2	C'-Flag	EcoRi-XhoI	Flag tagged via PCR-parent vector
57	C'flag-xMad1 MH1	pCS2	C'-Flag	XhoI-XbaI	Flag tagged via PCR
58	C'flag-xMad1 MH2	pCS2	C'-Flag	EcoRi-XhoI	deletion contruct from parent vector
59	C'flag-xMad1 linker+MH2	pCS2	C'-Flag	EcoRi-XhoI	deletion contruct from parent vector
60	C'flag-xMad1 linker+MH1	pCS2	C'-Flag	EcoRi-XhoI	deletion contruct from parent vector
61	flag-xsmad6	p3XFLAG-CMV	3xFlag	EcoRI-XbaI	interaction in cells
62	flag-xsmad7	p3XFLAG-CMV	3xFlag	EcoRI-XbaI	interaction in cells
63	myc-xmad1	pCS2-6myc	6xmyc	EcoRI-XbaI	
64	myc-xmad2	pCS2-6myc	6xmyc	XhoI-XbaI	
65	myc-xmad3	pCS2-6myc	6xmyc	XhoI-XbaI	
66	myc-xmad6	pCS2-6myc	6xmyc	EcoRI-XhoI	
67	myc-xmad7	pCS2-6myc	6xmyc	XhoI-XbaI	
68	myc-xmad6 MH1+linker	pCS2-6myc	6xmyc	EcoRI-XhoI	deletion contruct from parent vector 66
69	myc-xmad6 MH2	pCS2-6myc	6xmyc	EcoRI-XhoI	deletion contruct from parent vector 66