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Transcriptional Integration of Wnt and Nodal Signals in the Establishment of the Spemann Organizer

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Transcriptional Integration of Wnt and Nodal Signals in the Establishment of the Spemann Organizer

Abstract
Establishment of the vertebrate body axis requires the formation of the organizer domain during early embryogenesis. In amphibians, this domain is referred to as the Spemann Organizer and is essential for germ layer patterning and formation of the embryonic body axes. The Wnt and Nodal signaling pathways are both essential for organizer formation, but how these signals are integrated to influence gene expression in the organizer is largely unknown. The Wnt pathway activates expression of two transactivators, Siamois (Sia) and Twin (Twn), which mediate organizer formation downstream of Wnt. Expression of Sia or Twn is sufficient to induce an ectopic axis, suggesting they play an essential role in organizer formation. However, whether Sia/Twn play equivalent roles in organizer formation, or whether both are required for all aspects of organizer formation in not clear. Here, we report that knockdown of Sia/Twn together, but not individually, disrupts organizer gene expression and axis formation. We identify the Sia/Twn binding site within the promoter of the organizer gene *Goosecoid* (*Gsc*). Sia/Twn form homodimers and heterodimers through direct homeodomain interaction and both dimer forms are found at the endogenous *Gsc* promoter. The *Gsc* promoter also contains a Nodal responsive distal element, suggesting that Sia/Twn cooperate with Nodal signals in the transcription of *Gsc*. We find that Wnt and Nodal effectors synergize to activate transcription of three organizer genes, *Gsc*, *Cerberus* (*Cer*), and *Chordin* (*Chd*). Sia/Twn and the Nodal effectors FoxH1 and Smad2/3 occupy the promoters of these genes *in vivo* and their occupancy increases with active signaling from both pathways. This suggests that a complex consisting of Sia/Twn and Nodal effectors forms at organizer gene promoters. Consistent with this, p300 is recruited to organizer gene promoters in response to Sia/Twn or Nodal signals. Thus, Sia/Twn interact with Nodal effectors to regulate the spatial and temporal expression of organizer genes, suggesting a general mechanism for the regulation of organizer gene expression in the early embryo.

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TRANSCRIPTIONAL INTEGRATION OF WNT AND NODAL SIGNALS IN THE ESTABLISHMENT OF THE SPEMANN ORGANIZER

Christine D. Reid

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Dedication

To the memory of my Grandmother, Frances Viola Reeser Seaton, who was my first teacher. She taught me the joy of both teaching and learning and encouraged my interest in science from a very young age.

To my parents, Ronald and Janet Reid, who have consistently impressed upon me that I can be whatever I want to be. And upon hearing that their only child was moving across the country to pursue an advanced degree, only had vast amounts of support, kindness and patience.

To my closest friend, Randi Friday, who helped me through the toughest times with copious amounts of humor and gentle reminders of my goals and my abilities.

And lastly, to my future husband, Alexander Brown, whose pursuit of science every day has consistently inspired me to be the best scientist I can be.

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Abstract

Transcriptional Integration of Wnt and Nodal Signals in the Establishment of the Spemann Organizer

Christine D. Reid

Daniel S. Kessler

Establishment of the vertebrate body axis requires the formation of the organizer domain during early embryogenesis. In amphibians, this domain is referred to as the Spemann Organizer and is essential for germ layer patterning and formation of the embryonic body axes. The Wnt and Nodal signaling pathways are both essential for organizer formation, but how these signals are integrated to influence gene expression in the organizer is largely unknown. The Wnt pathway activates expression of two transactivators, Siamois (Sia) and Twin (Twn), which mediate organizer formation downstream of Wnt. Expression of Sia or Twn is sufficient to induce an ectopic axis, suggesting they play an essential role in organizer formation. However, whether Sia/Twn play equivalent roles in organizer formation, or whether both are required for all aspects of organizer formation in not clear. Here, we report that knockdown of Sia/Twn together, but not individually, disrupts organizer gene expression and axis formation. We identify the Sia/Twn binding site within the promoter of the organizer gene *Goosecoid* (*Gsc*). Sia/Twn form homodimers and heterodimers through direct homeodomain interaction and both dimer forms are found at the endogenous *Gsc* promoter. The *Gsc* promoter also contains a Nodal responsive distal element, suggesting that Sia/Twn cooperate with Nodal signals

in the transcription of *Gsc*. We find that Wnt and Nodal effectors synergize to activate transcription of three organizer genes, *Gsc*, *Cerberus* (*Cer*), and *Chordin* (*Chd*). Sia/Twn and the Nodal effectors FoxH1 and Smad2/3 occupy the promoters of these genes *in vivo* and their occupancy increases with active signaling from both pathways. This suggests that a complex consisting of Sia/Twn and Nodal effectors forms at organizer gene promoters. Consistent with this, p300 is recruited to organizer gene promoters in response to Sia/Twn or Nodal signals. Thus, Sia/Twn interact with Nodal effectors to regulate the spatial and temporal expression of organizer genes, suggesting a general mechanism for the regulation of organizer gene expression in the early embryo.

Table of Contents

List of Tables

List of Figures

Chapter 1 : Formation and Function of the Spemann Organizer

1.1. Summary

Embryonic development consists of a complex interplay of distinct signaling pathways and intricate tissue movements. Patterning and specification of the embryonic axes is largely dependent on the formation of the organizer domain, termed the node in mouse and chick, the shield in zebrafish, and the Spemann-Mangold organizer in amphibians. The organizer acts as a source of inhibitors, creating a gradient of signaling activity across the embryo to direct germ layer patterning and axis formation during early development. The organizer forms in response to two pathways, the Wnt pathway and the Nodal pathway. These two pathways are responsible for the expression of a number of genes that both directly and indirectly contribute to organizer function. The Wnt pathway activates expression of two transcriptional activators, *Siamois* (*Sia*) and *Twin* (*Twn*) that mediate organizer formation downstream of Wnt signals. Expression of Sia or Twn is sufficient to induce a fully functional organizer, but how Sia/Twn regulate transcription of organizer genes is not well understood. The Nodal pathway activates gene expression through maternally deposited stores of the effectors FoxH1 and Smad2/3. While several organizer genes, including *Goosecoid* (*Gsc*), *Cerberus* (*Cer*) and *Chordin* (*Chd*) require inputs from both the Wnt and Nodal pathways for proper expression. The *Gsc* promoter contains Wnt and Nodal responsive elements, suggesting that inputs from these two pathways may be integrated at the level of transcription. This study is focused on identifying the role of Sia and Twn in organizer formation, both at the level of organizer gene promoters and in the embryo, and determining how Sia/Twn and Nodal signals are integrated at the level of transcription to induce organizer formation.

1.2. Embryonic Development

At the time of egg laying, the amphibian egg has localized mRNA and proteins within both the animal and vegetal hemispheres (reviewed in Heasman, 2006). These localized messages and proteins contribute to patterning, as their localization allows for limited or concentrated signaling activity (reviewed in Heasman, 2006). The maternal stores of proteins and mRNAs control signaling and patterning during the early cleavage stages of development, when the cell cycle is shortened to an S-phase for DNA synthesis and an M phase for mitosis (Newport and Kirschner, 1982a). The sperm enters the embryo in the animal hemisphere and sets off a series of event that leads to the formation of the presumptive dorsal side of the embryo, where the Spemann organizer will form (Black and Gerhart, 1985). The organizer is a small region of the embryo that is essential for patterning the emerging germ layers and for providing cues to form the vertebrate axes (reviewed in Harland and Gerhart, 1997). Once fertilized, the cortex of the egg cytoplasm undergoes a microtubule-dependent rotation away from the site of sperm entry (Scharf and Gerhart, 1983). This cortical rotation moves mRNAs and proteins that had been localized to the vegetal cortex of the embryo to the future dorsal side of the embryo, opposite the site of sperm entry (Holwill et al., 1987; Scharf and Gerhart, 1983). If this microtubule dependent movement is disrupted by ultraviolet light, the gene expression that would normally occur on the future dorsal side of the embryo now transiently occurs in the vegetal pole of the embryo, disrupting patterning and resulting in an embryo lacking dorsal structures (Scharf and Gerhart, 1983).

During the first twelve cell divisions of embryonic development, stores of maternal proteins and mRNAs are utilized for early signaling and patterning events (Newport and Kirschner, 1982a). Several zygotic genes are actively expressed, and

important patterning cues are initiated (Yang et al., 2002), but the global zygotic genome is largely silent (Newport and Kirschner, 1982a). The embryonic cells are thought to sense the increasing nuclear to cytoplasmic ratio, and after 12 divisions, the cell cycle slows to include G1 and G2 phases (Newport and Kirschner, 1982b). This time point is referred to as the mid-blastula transition (MBT), at which point the zygotic genome begins to express multiple genes, maternally contributed proteins and mRNAs are degraded, and the cells become more motile in preparation for gastrulation (Duval et al., 1990; Newport and Kirschner, 1982a).

Prior to MBT, the cells of the developing embryo, if transplanted to another embryo, can contribute to all germ layers, suggesting that all cells within the early embryo are totipotent (Heasman et al., 1984). As the embryo ages, cell fates become more restricted. A gastrula stage vegetal cell contributes only to endodermal tissues (Heasman et al., 1984; Wylie et al., 1987), while a gastrula stage animal cell contributes to ectodermal tissues (Snape et al., 1987). These experiments suggest that cells are no longer totipotent by the time of gastrulation, and that the ability of cells to form particular germ layers has been restricted. After gastrulation, the germ layers have been specified: the ectoderm, fated to form the skin and the nervous system; the mesoderm, forming the skeletal muscle, heart and blood; and the endoderm, which forms the internal organs, including the gut, lungs, liver and pancreas (reviewed in Heasman, 2006). At gastrulation, cells begin to involute at the dorsal marginal zone, leading to the elongation of the embryo as cells intercalate to form the elongated vertebrate body (reviewed in Harland and Gerhart, 1997). The site of gastrulation serves to specify cell fates later in development. Cells that form the anterior and dorsal structures of the embryo are located near the site of initiation of gastrulation, while those cells that form ventral or

posterior structures are located farther away from that site (reviewed in De Robertis et al., 2000; Harland and Gerhart, 1997; Niehrs, 2004). What establishes this global pattern is the organizer, which forms just above the site of the initiation of gastrulation. As cells pass through or near the organizer during gastrulation, they are exposed to varying degrees of signaling activity, which, when coupled with a changing competence of cells to respond to such signals, are thought to confer positional information for tissue patterning (reviewed in Harland and Gerhart, 1997). The organizer not only provides anterior/posterior positional information, likely by cells interpreting their own distance from the organizer, but it also confers dorsal/ventral information, and left/right pattern as well (reviewed in Harland and Gerhart, 1997; Wood, 1997). The organizer appears to be non-homogenous, as the anterior domain of the organizer induces head structures, while the posterior domain of the organizer induces trunk structures, demonstrating that the organizer can be further sub-divided into the head organizer domain and the trunk organizer domain (Zoltewicz and Gerhart, 1997).

The organizer was discovered in 1924 in classic experimentation performed by Hilde Mangold and Hans Spemann (reviewed in Hamburger, 1988). Transplantation of the dorsal blastopore lip of a gastrulating newt embryo onto the ventral side of a host embryo resulted in ectopic axis formation (Hamburger, 1988). The resulting secondary, or “siamese”, axis consisted almost entirely of host tissue (Gimlich and Cooke, 1983; Smith and Slack, 1983), suggesting that this small region of tissue, eventually termed the Spemann-Mangold organizer, contains inductive cues that organize surrounding tissues into a complete body axis (reviewed in Hamburger, 1988). This revolutionary discovery led to a Nobel prize, and invigorated the field of developmental embryology (reviewed in Hamburger, 1988). It was found that the organizer itself performs three

distinct functions. Firstly, the organizer promotes morphogenesis of the organizer cells themselves with distinct movements attributed to the head and trunk organizer cells, respectively. These morphogenic movements promote cell movement in surrounding tissues that receive various signaling cues as the cells move. Secondly, the organizer self-differentiates into the mesodermal tissues of the notochord and prechordal plate and the endodermal derivatives of the pharyngeal tissues and anterior gut. And thirdly, the organizer induces surrounding germ layers to form dorsal tissues, including dorsal mesoderm, neuralized ectoderm and anterior endoderm. These signals occur non-cell autonomously, suggesting that cells of the organizer secrete specific dorsalizing factors into surrounding tissues (reviewed in Harland and Gerhart, 1997).

1.3. The Molecular Mechanisms of Organizer Function

Since the discovery of the organizer, work has focused on identifying the molecular components that contribute to organizer formation and function. Multiple organizer genes have been identified, and most, if not all, of these genes function as signaling inhibitors, both intracellular and extracellular, which are thought to temper activity of a number of signaling pathways including the Wnt, Nodal and BMP pathways. Extracellular organizer genes include *Dickkopf-1* (*Dkk-1*) and *Crescent*, inhibitors of the Wnt pathway (Glinka et al., 1998; Pera and De Robertis, 2000; Shibata et al., 2000), *Chordin* (*Chd*), *Noggin* (*Nog*) and *Follistatin* (*Xfs*), inhibitors of BMP signaling (Fainsod et al., 1997; Piccolo et al., 1996; Zimmerman et al., 1996), *Lefty*, inhibitor of Nodal signaling (Cheng et al., 2000), and *Cerberus*, inhibitor of all three signaling pathways (Piccolo et al., 1999). Intracellular organizer genes include the transcriptional repressor *Gsc*, which directly suppresses expression of *Wnt8* within the organizer domain (Yao

and Kessler, 2001).

Several of these factors have been to shown to be essential for organizer function. For example, triple knockdown of *Chd*, *Nog* and *Xfs* in amphibian embryos results in the failure to form the dorsal organizer and an expansion of ventral tissues, implying that BMP inhibition is essential for dorsal ventral patterning (Khokha et al., 2005). Similarly, *Chd*$^{-/-}$;*Nog*$^{-/-}$ mice lack anterior structures, suggesting a critical role for these two BMP antagonists in patterning the mouse embryo (Bachiller et al., 2000). Overexpression of *Cer* in the amphibian embryos is necessary and sufficient for head formation (Bouwmeester et al., 1996; Kuroda et al., 2004), but deletion of *Cerberus-like* in the mouse has no effect on head formation (Simpson et al., 1999), suggesting that *Cer* may perform overlapping or redundant tasks with other organizer genes in mouse. In *Xenopus*, knockdown of *Gsc* expression leads to reduced dorsal structures and an expansion of ventral tissues (Sander et al., 2007). However, *Gsc*$^{-/-}$ mice have normal gastrulation and node formation, yet the mutation is neonatal lethal due to craniofacial abnormalities that arise later in development (Rivera-Perez et al., 1995; Yamada et al., 1995). Mice mutant for *Hnf-3*β (*Foxa2*) display dorsal ventral patterning defects in the neural tube, yet maintain anterior posterior patterning in the embryo, suggesting that *Hnf-3*β is important in some functions of the mouse node (Ang and Rossant, 1994). *Gsc*$^{-/-}$; HNF-3$\beta^{+/-}$ mice display severe anterior/posterior patterning defects, with a loss of forebrain, anterior gut and dorsal mesoderm derivatives (Filosa et al., 1997), suggesting that in the mouse *Gsc* may function redundantly with *Hnf-3*β in node formation. While many molecular components of the organizer have been identified, several play multiple or redundant roles during organizer function. As such, specific temporal and spatial expression patterns of these molecular components are likely important to the function of

the organizer throughout gastrulation.

1.4. The Role of the Wnt Pathway in Organizer Formation

The canonical Wnt pathway signals through multiple Wnt ligands that bind to Frizzled receptors and Low Density Lipoprotein receptor (LRP) co-receptors, which act to stabilize β-catenin, which translocates to the nucleus to activate dorsal gene expression (reviewed in Weaver and Kimelman, 2004). In the early cleavage stages of development, nuclear β-catenin is observed solely on the future dorsal side of the embryo, suggesting that Wnt pathway activation is a consequence of cortical rotation (Schneider et al., 1996). Wnt11 mRNA is localized to the vegetal hemisphere of the egg, and is required for dorsal development (Tao et al., 2005). Similarly, inhibition of the Wnt pathway by knockdown of β-catenin, Frizzled-7, or LRP6 results in loss of organizer gene expression and failure to form dorsal structures (Heasman et al., 1994; Sumanas et al., 2000; Yang et al., 2002). Taken together, the maternal Wnt pathway is required for organizer formation during embryogenesis.

The Wnt pathway is also important in regulating formation of the organizer in other vertebrates. *Wnt3a*$^{-/-}$ mice display a disruption of node formation and lack a primitive streak and mesodermal derivatives (Liu et al., 1999). Deletion of the Wnt effector *β-catenin* results in mice that fail to form a mesodermal germ layer and subsequently do not gastrulate (Haegel et al., 1995), suggesting an early role for Wnt signaling in mammalian development. In the zebrafish, loss of *TCF3* results in reduction of anterior head formation, suggesting that *TCF3* acts as a repressor of Wnt signaling during organizer formation (Kim et al., 2000). Similarly in the mouse, *TCF3* is a repressor of Wnt signaling during early embryogenesis, as *TCF3*$^{-/-}$ embryos often display

duplicated axes, expansion of dorsal structures including the notochord, and defects in anterior-posterior patterning (Merrill et al., 2004). In the chick, nuclear β-catenin is localized to an area where the node will form (Roeser et al., 1999). Ectopic activation of the Wnt pathway through LiCl treatment leads to anteriorized embryos, suggesting that Wnt plays an essential role in the formation of the chick node as well (Roeser et al., 1999). Taken together, the Wnt signaling pathway plays an essential role in the formation of the vertebrate organizer, as Wnt activity is required for axis formation and patterning in multiple model systems.

1.5. The *Xenopus* Wnt Effectors Siamois and Twin

The homeodomain transcription factors Sia and Twn were discovered in screens for the molecular components of the organizer (Laurent et al., 1997; Lemaire et al., 1995). Sia and Twn are expressed in the amphibian embryo at the onset of MBT in an area fated to become the organizer (Laurent et al., 1997; Lemaire et al., 1995). Expression of either Sia or Twn on the ventral side of the embryo results in an ectopic axis, consisting of both head and trunk tissue, suggesting that Sia and Twn can induce formation of the complete organizer (Laurent et al., 1997; Lemaire et al., 1995). Sia and Twn are highly similar, sharing nearly 90% identity within the homeodomain (Laurent et al., 1997). The third helix of the homeodomain, which makes direct contacts with DNA targets, shares 100% identity between Sia and Twn, suggesting that they may share transcriptional targets (Laurent et al., 1997; Wilson et al., 1995). There is also high homology with three other domains N-terminal to the homeodomain, termed the A, B, and C regions (Fig. 1.1) (Laurent et al., 1997), but the function of these regions is unknown. Because Sia and Twn expression so clearly and reliably mimicked organizer transplantation, a search for

Figure 1.1 Siamois and Twin Protein Structure and Conservation

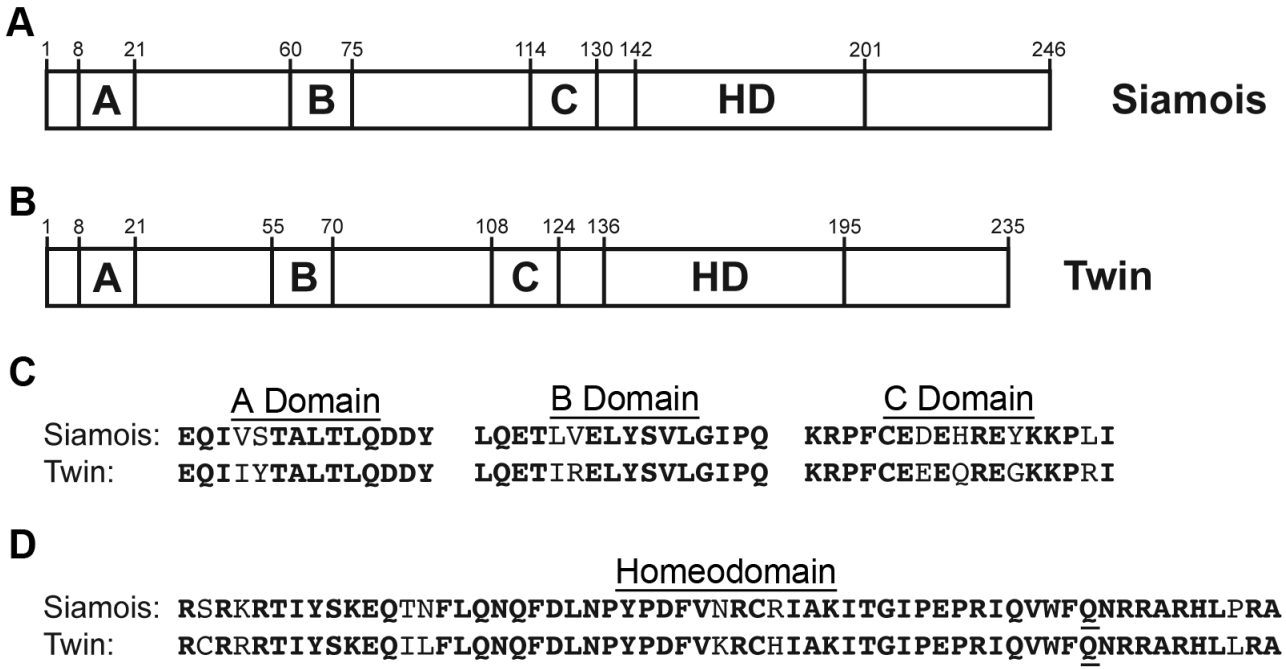

Figure 1.1: Siamois and Twin Protein Structure and Conservation

(A-B) Schematic of the Siamois and Twin proteins, highlighting the conserved N-terminal domains termed the A, B and C domains, and the location of the homeodomain (HD). Amino acid number is indicated on the top of the schematic. (C) A comparison of the sequence of the conserved A, B and C domains of Siamois and Twin, respectively. Conserved residues are in bold. (D) A comparison of the homeodomain sequence of Sia and Twn. Conserved residues are in bold. The underlined glutamine (Q) (position 191 in Sia, position 185 in Twn) in the homeodomain is critical for recognition of DNA sequence (Wilson et al., 1995). Mutation of this residue results in a loss of DNA binding activity (Kessler, 1997). Schematics and sequence adapted from (Laurent et al., 1997).

vertebrate orthologs was undertaken. However, after many years, mouse, chick, or zebrafish orthologs of Sia or Twn have yet to be identified, suggesting that vertebrate organizers may have different molecular requirements for organizer gene expression. Sia and Twn are homeodomain proteins, but do not have other definable domains, making a search for orthologs more difficult. Defining functional domains within Sia and Twn is essential for understanding how Sia and Twn control organizer formation and also in identifying potential orthologs that function as Sia/Twn in organizer formation.

Expression of Sia or Twn rescues axis formation in UV-ventralized embryos, suggesting that Sia and Twn may function downstream of the maternal Wnt pathway (Laurent et al., 1997; Lemaire et al., 1995). Both Sia and Twn are induced in response to active Wnt signals, and consensus LEF/TCF sites within both the Sia and Twn promoters are important for their expression (Brannon and Kimelman, 1996; Carnac et al., 1996; Crease et al., 1998; Fan et al., 1998; Labbe et al., 2000; Nelson and Gumbiner, 1998; Nishita et al., 2000). Cells are competent to respond to Sia activity during the blastula stage, suggesting that Sia is most active during this time (Kodjabachian and Lemaire, 2001). The cells of the embryo are responsive to Sia activity around MBT, as the ability of Sia to induce a complete ectopic axis, and thus the formation of a fully functional organizer, is rapidly lost shortly thereafter (Kodjabachian and Lemaire, 2001). Inhibition of Sia activity prevents Wnt-mediated ectopic axis formation, suggesting that Sia functions downstream of maternal Wnt pathway activation (Fan and Sokol, 1997; Kessler, 1997). Taken together, Sia and Twn are sufficient to induce the formation of a fully functional organizer downstream of Wnt, and likely play an important role in organizer formation during early embryogenesis.

1.6. The Role of Siamois and Twin in Organizer Formation

The organizer performs three distinct tasks: the direct autonomous differentiation of organizer tissue, the induction of morphogenesis, not only in the organizer itself, but also in the surrounding tissue, and the release of inductive cues which result in the dorsalization of the mesoderm, the neuralization of the ectoderm and the anteriorization of the endoderm (reviewed in Harland and Gerhart, 1997). Ectodermal tissue expressing Sia can dorsalize ventral mesoderm in a non-cell autonomous manner, suggesting that Sia may, directly or indirectly, induce expression of secreted dorsalizing agents (Carnac et al., 1996). Sia expression in ectodermal tissue results in formation of cement gland, an anterior tissue, and neural tissue, while expression of Sia in the ventral marginal zone induces dorsal axial tissues and a secondary site of gastrulation (Carnac et al., 1996). These properties of Sia-expressing tissues are strikingly similar to the properties of organizer tissue itself, suggesting that Sia expression is sufficient for organizer formation (Carnac et al., 1996; Dale and Slack, 1987).

Because of the high level of similarity between Sia and Twn, not only in function but also in structure, it was assumed that Sia and Twn function redundantly (Laurent et al., 1997). To determine the requirement of Sia and Twn in organizer formation, fusion proteins containing the homeodomain of Sia and the Engrailed repressor domain (Eng-Sia) were constructed, with the goal that these constructs would repress both Sia and Twn targets alike (Fan et al., 1998; Kessler, 1997). Dorsal expression of Eng-Sia completely blocks organizer gene expression and formation of dorsal embryonic structures (Fan and Sokol, 1997; Kessler, 1997). This phenotype is strikingly similar to the phenotypes observed upon removal of the organizer by extirpation, UV irradiation, or knockdown of β-catenin (Heasman et al., 1994; Scharf and Gerhart, 1983; Stewart and

Gerhart, 1990), suggesting that Sia, and perhaps Twn, are required for organizer formation.

However, subsequent knockdown studies of Sia and Twn did not reflect this hypothesis. Knockdown of Sia and Twn by morpholino oligonucleotide resulted in a ventralized embryo lacking the anterior-most structures, the forebrain and midbrain, but dorsal structures, including the notochord, were still present (Ishibashi et al., 2008). In contrast, dorsal expression of Eng-Sia resulted in ventralized embryos, lacking both anterior and dorsal structures (Fan and Sokol, 1997; Kessler, 1997). This raises the possibility that Eng-Sia may repress genes that are normally not targets of Sia or Twn, or that knockdown of Sia and Twn in these studies was not complete. If Sia/Twn are indeed required for the formation of the fully functional organizer, then a partial knockdown of Sia/Twn activity may result in increasingly more severe anterior truncations, as organizer formation is restricted. However, if Sia/Twn are required only for the formation of the head organizer, increasing inhibition of Sia/Twn activity would only affect the anterior-most tissues of the head. Interestingly, the phenotype observed in Sia/Twn knockdown embryos is reminiscent of the phenotypes obtained when small regions of the organizer are extirpated from an embryo. As more organizer tissue is removed from the embryo, more anterior tissue is lost (Stewart and Gerhart, 1990), suggesting that incomplete knockdown of Sia/Twn may cause a partial defect in organizer formation and function. These observations offer two possibilities for the role of Sia/Twn in organizer formation. Sia and Twn could be necessary for formation of the entire, fully functional organizer, or could play a more limited role in the formation of the head organizer. Given these distinct possibilities, further work is required to determine the exact role of Sia and Twn in organizer formation.

1.7. Transcriptional Targets of Siamois and Twin

As transcriptional activators expressed prior to organizer formation, Sia and Twn likely influence organizer formation by direct control of the expression of organizer genes. Sia expression in ventral mesoderm induces expression of multiple organizer genes, including *Gsc*, *Chd*, *Cer*, *Nog*, *Frzb1*, *Xfs*, *Xlim1*, *Otx2*, and *Xnot2* (Carnac et al., 1996; Engleka and Kessler, 2001; Fan and Sokol, 1997; Kessler, 1997; Kodjabachian and Lemaire, 2001). Sia-induced expression of *Cer*, *Frzb1* and *Xlim1*, which are head organizer genes, is dependent on active Nodal signaling (Engleka and Kessler, 2001), suggesting that Sia may cooperate with Nodal in the expression of some organizer genes. To date, the promoter regions of *Gsc* and *Cer* have been identified (Fig. 1.2). The *Gsc* promoter contains two elements, a Wnt responsive proximal element (PE) and a Nodal responsive distal element (DE), which are separated by approximately 50 base pairs (Fig. 1.2A) (Watabe et al., 1995). Twn was shown to protect a region of the *Gsc* PE that contains a consensus homeodomain binding site (Laurent et al., 1997). The high level of similarity between the Sia and Twn homeodomain sequence suggests that Sia likely mediates *Gsc* expression through the same region (Laurent et al., 1997). The *Cer* promoter contains multiple homeodomain binding sites that mediate response to Sia, Mix.1, Otx2 and Xlim-1 (Fig. 1.2B) (Yamamoto et al., 2003).

Several questions remain about the regulation of organizer gene expression. While Sia and Twn clearly play a role in organizer gene expression, it is less clear how signals from other pathways are involved in organizer gene expression. Sia and Twn could be part of a common mechanism of organizer gene expression or each organizer gene could be regulated uniquely. Similarly, it remains unclear how the timing and regional specificity of organizer gene induction is regulated. Sia and Twn are expressed

Figure 1.2 Schematic of the *Goosecoid* and *Cerberus* promoters

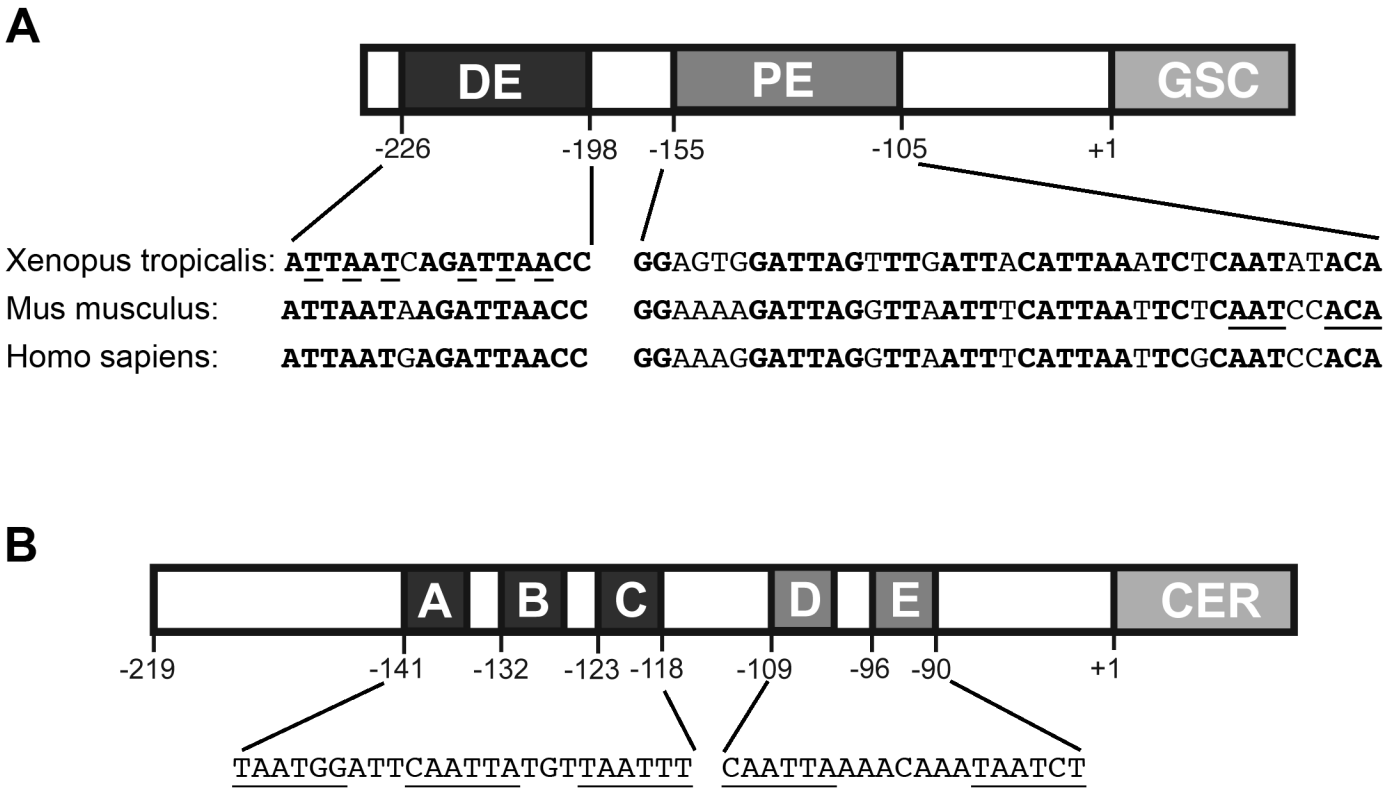

Figure 1.2: Schematic of the *Goosecoid* and *Cerberus* promoters.

(A) Schematic of the *Gsc* promoter, indicating the Nodal-responsive Distal Element (DE) and the Wnt responsive Proximal Element (PE). Below the schematic is the sequence of the *Xenopus*, mouse and human regulatory regions of the *Gsc* promoter. Conserved bases are indicated in bold. In the *Xenopus* DE, underlined bases represent sequence identified as important for the response to Nodal. In the mouse PE, underlined sequence indicates the identified FoxH1 binding site (Labbe et al., 1998). Adapted from (Bae et al., 2011; Watabe et al., 1995) (B) Schematic of the *Cerberus* promoter region, containing five homeodomain binding sites, termed A, B, C, D, and E. The sequence of the sites is indicated below the schematic, with the A, B, C, D and E sites underlined, respectively. The A, B and E sites are most important for dorsal domain expression of Cer. The B site was shown to be the main site through which Xlim1 signals. The A, B and C domains are essential for response to Nodal and Wnt. Site E is a bicoid type binding site, thought to be utilized by Otx2. Adapted from (Yamamoto et al., 2003)

within the organizer domain at blastula stage, and Sia activity is highest during this early stage (Kodjabachian and Lemaire, 2001; Laurent et al., 1997; Lemaire et al., 1995), suggesting that Sia/Twn are acting shortly after MBT to influence gene expression and organizer formation. However, other transcription factors, such as Otx2, are expressed at the gastrula stage (Blitz and Cho, 1995). And yet other transcription factors, like the Nodal effector Mix.1, have broad, endodermal expression (Rosa, 1989), implying that other factors must be involved in restricting organizer gene expression to the future dorsal side of the embryo. Yet all of these transcription factors are involved in the expression of the organizer gene *Cer* (Yamamoto et al., 2003), suggesting that different transcription factors may induce organizer gene expression at different time points and perhaps in different manners. Whether these signals interact to influence gene expression patterns is not well understood.

1.8. The Role of Nodal Signaling in Organizer Formation

Nodal ligand binding to a Type II activin-like Ser/Thr kinase receptor activates the Type I receptor. These receptors phosphorylate the intracellular receptor Smads, Smad2 and Smad3, which complex with Smad4 to enter the nucleus. This Smad complex interacts with DNA bound transcription factors such as FoxH1 and the Mix family of homeodomain proteins, to affect gene transcription (reviewed in Schier and Shen, 2000). While only one Nodal ligand is found in mouse (Conlon et al., 1991; Conlon et al., 1994; Iannaccone et al., 1992), the *Xenopus* embryo contains several Nodal ligands, termed Xenopus nodal related, or Xnrs (Jones et al., 1995; Takahashi et al., 2000).

The Nodal pathway regulates organizer formation, induction, and patterning of the endodermal and mesodermal germ layers (reviewed in De Robertis et al., 2000).

Two maternally supplied mRNAs, Vg1 and VegT, are localized to the vegetal hemisphere of the egg (Weeks and Melton, 1987; Zhang and King, 1996). Vg1 acts as a TGF-β ligand, and VegT is a T-box transcription factor; both are required for axis formation (Birsoy et al., 2006; Kofron et al., 1999; Xanthos et al., 2001). Knockdown of VegT results in a loss of *Xnr* gene expression and a failure to form endodermal derivatives. This can be rescued by expression of any of a number of *Xnr* ligands, suggesting that the role of maternal VegT is to promote expression of zygotic Nodal (Kofron et al., 1999; Xanthos et al., 2001). Knockdown of Vg1 results in a loss of organizer gene expression, and embryos develop with reduced anterior structures (Birsoy et al., 2006). In concert with β-catenin and the maternal Wnt signaling pathway, these factors activate expression of the *Xnrs* (Agius et al., 2000).

Several Xnr ligands are expressed in the early embryo, including *Xnr1*, *Xnr2*, *Xnr5* and *Xnr6* (Jones et al., 1995; Takahashi et al., 2000). With such a large number of likely redundant Nodal ligands acting in the early embryo, it has been difficult to work out the exact role of each ligand in germ layer patterning and organizer formation (reviewed in De Robertis et al., 2000). Blocking Nodal activity by expression of a dominant negative receptor results in a loss of organizer gene expression (Watanabe and Whitman, 1999). Expression of a dominant-negative *Xnr5* delays gastrulation and causes severe anterior defects. Interestingly, the expression of other *Xnrs* is reduced in these embryos, suggesting that early Nodal signaling is essential for persistence of later Nodal signaling (Onuma et al., 2002). Expression of *Cer*-short, a form of *Cerberus* that specifically inhibits Nodal signaling, results in a loss of mesodermal and organizer gene expression (Agius et al., 2000), suggesting that Nodal plays an important role in the formation of the mesodermal germ layer and in organizer formation.

The Nodal pathway signals through phosphorylated Smad2/3 and a DNA-bound transcription factor FoxH1, which form a complex at target genes to affect transcription (Chen et al., 1996). In the frog, phosphorylation of Smad2 can be detected as early as the 1000 cell stage, suggesting the Nodal pathway is active prior to the MBT (Schohl and Fagotto, 2002; Skirkanich et al., 2011). Indeed, *Xnr5* and *Xnr6* are expressed prior to the MBT in response to maternal Wnt signaling (Blythe et al., 2009; Yang et al., 2002), further suggesting that the Nodal pathway actively signals prior to MBT. Expression of a dominant negative Smad2 mutant during early embryogenesis results in a loss of dorsoanterior structures (Hoodless et al., 1999). Knockdown of maternal FoxH1 results in a loss of organizer gene expression, with embryos lacking anterior structures (Kofron et al., 2004a).

Nodal is also essential for the formation of other vertebrate organizers. In the chick, expression of Nodal is both sufficient and necessary for formation of the primitive streak (Bertocchini and Stern, 2002; Mitrani et al., 1990). Loss of Nodal signaling in zebrafish, via mutations in the nodal ligands *cyclops* and *squint* or maternal zygotic mutations in the Nodal co-receptor *one-eyed pinhead* (*oep*), results in a loss of mesoderm and severe disruption in anterior/posterior and dorsal/ventral patterning (Dougan et al., 2003; Gritsman et al., 2000; Gritsman et al., 1999; Thisse et al., 2000). In the mouse, Nodal plays a critical role in primitive streak formation, as embryos lacking Nodal have severe axial defects and lack embryonic mesoderm (Conlon et al., 1991; Conlon et al., 1994; Vincent et al., 2003).

In the zebrafish, knockdown of FoxH1 disrupts cell movement and gastrulation, suggesting that FoxH1 and Nodal signaling may promote morphogenesis within the organizer (Pei et al., 2007). *FoxH1*$^{-/-}$ mice display defects in the primitive streak and fail

to form the node and its derivatives such as notochord and prechordal plate mesoderm (Hoodless et al., 2001). *Smad2*$^{-/-}$*; Smad3*$^{-/-}$ mouse embryos fail to form any mesodermal germ layers and do not gastrulate (Dunn et al., 2004). Taken together, the Nodal effectors FoxH1 and Smad2/3 play an essential role in organizer formation during vertebrate embryogenesis.

1.9. The Regulation of Organizer Genes by Wnt and Nodal

While multiple organizer genes have been identified, the specific transcriptional inputs controlling expression of organizer genes are not well understood. Overexpression of Wnt or Nodal induces expression of multiple organizer genes, but the mechanisms of transcriptional regulation is not well understood (for review, see (De Robertis, 2006; De Robertis et al., 2000; Harland and Gerhart, 1997; Niehrs, 2004). Perhaps the best characterized organizer gene promoter region is that of *Gsc*, a transcriptional repressor expressed within the organizer domain at gastrulation (Blumberg et al., 1991; Watabe et al., 1995). The *Xenopus Gsc* promoter contains two distinct elements, a Proximal Element (PE) responsive to Wnt signals, and a Distal Element (DE) responsive to Nodal signals (Watabe et al., 1995). The PE and DE are highly conserved with the mouse *Gsc* promoter, suggesting that *Gsc* may be regulated by Wnt and Nodal inputs in other vertebrate species (Watabe et al., 1995). However, the PE does not contain consensus TCF/LEF sites to mediate a direct signal from maternal Wnt signals, suggesting that factors downstream of Wnt regulate *Gsc* expression. Instead, it contains several homeodomain binding sites that bind Twn (Laurent et al., 1997) and presumably Sia. Both Sia and Twn activate expression of a *Gsc* luciferase reporter, and removal of the PE prevents activation, suggesting that Sia/Twn activate

expression of *Gsc* through direct interaction with the PE (Kessler, 1997; Laurent et al., 1997). However, whether the conserved homeodomain sites within the PE mediate Sia/Twn mediated expression of *Gsc* is unknown.

The effectors that mediate Nodal signaling in *Gsc* expression include FoxH1 and the homeodomain effectors Mixer and Milk (Chen et al., 1996; Ecochard et al., 1998; Henry and Melton, 1998). FoxH1 is maternally deposited throughout the embryo, and acts to induce expression of both mesodermal and endodermal genes (Chen et al., 1996; Kofron et al., 2004a). Maternal knockdown of FoxH1 reduces expression of *Gsc* at late blastula and early gastrula stages (Kofron et al., 2004a), suggesting that FoxH1 plays an essential role in the initiation of *Gsc* expression. Indeed, mammalian FoxH1 mediates TGF-β signaling through a conserved binding site within the *Gsc* promoter (Labbe et al., 1998). However, this site is within the Wnt responsive PE, downstream of the homeodomain binding sites (Labbe et al., 1998). This region of the promoter is not sufficient to respond to activation of Nodal signaling (Watabe et al., 1995), suggesting that FoxH1 might either have multiple binding sites within the *Gsc* promoter or that FoxH1 cooperates with other Nodal effectors in the expression of *Gsc*.

While FoxH1 plays a role in the early response to Nodal signaling, the roles that zygotically expressed Mixer and Milk play in organizer formation are unclear. Mixer is broadly expressed in endodermal cells, and inhibition of Mixer function results in a loss of endodermal gene expression, but not organizer gene expression (Henry and Melton, 1998). Further study of the knockdown of Mixer expression found that Mixer seems to have a gene-specific effect on endodermal and mesodermal gene expression. For example, loss of Mixer results in an increase in *Cer* expression, suggesting that Mixer may play a role in negatively regulating expression of some organizer genes (Kofron et

al., 2004b). Similarly, Milk seems to play a role in endoderm formation by repressing expression of mesodermal genes. Overexpression of Milk leads to a loss of dorsal organizer gene expression, including *Gsc*, suggesting that Milk likely negatively regulates organizer gene expression (Ecochard et al., 1998). Nodal signals are thought to exist in a gradient across the embryo, with highest activity on the future dorsal side of the embryo, within the vegetal hemisphere, and lowest activity on the future ventral marginal zone (reviewed in Kimelman, 2006). Levels of Nodal morphogen are though to correspond to the induction of distinct cell types within the mesodermal and endodermal germ layers (Green et al., 1992), but the transcriptional changes associated with a Nodal gradient are not well understood. Varying levels of Nodal could lead to the activation of multiple transcription factors, leading to altered gene expression within the germ layers. Whether Mixer, Milk, or FoxH1 contribute to interpretation of such a gradient remains to be determined.

However, both Milk and Mixer can form a complex with Smad2 at the DE of the *Gsc* promoter (Germain et al., 2000). Mutation of the homeodomain binding sites within the DE prevents activin induced transcriptional activity, suggesting that Mixer and Milk may mediate *Gsc* expression through the DE (Germain et al., 2000). It should be noted that these experiments were performed in cell culture and the *in vivo* knockdown and overexpression results suggest that Mixer and Milk may initially repress *Gsc* (Ecochard et al., 1998; Kofron et al., 2004b). Careful testing of these hypotheses will have to be performed in the embryo to determine the precise roles of Mixer and Milk in organizer gene expression. In all likelihood, several transcription factors mediate endogenous *Gsc* expression, as different protein complexes were found to form at the *Gsc* promoter in zebrafish during blastula and gastrula stages (McKendry et al., 1998). Mixer, Milk and

other Nodal pathway effectors may interact to produce temporally and spatially distinct patterns of gene expression in the early embryo.

The organizer gene *Cer* is also regulated by Wnt and Nodal inputs (Yamamoto et al., 2003). Knockdown of FoxH1 results in reduced expression of *Cer* at early stages (Kofron et al., 2004a), and Sia and Mix.1 synergistically enhance *Cer* expression in ectodermal explants (Lemaire et al., 1998). *Cer* expression is regulated by a group of homeodomain transcription factors, including Sia, Xlim-1, Xotx2 and Mix.1 (Yamamoto et al., 2003). These factors act through a set of homeodomain binding sites located proximal to the start site of transcription (Yamamoto et al., 2003). However, it remains unclear how these transcription factors interact to induce *Cer* expression, as they are expressed at different time points in development. *Sia* is expressed at the MBT (Blythe et al., 2010), but *Mix.1* is not expressed until gastrulation (Henry and Melton, 1998), when *Cer* expression is initiated (Bouwmeester et al., 1996). Similarly, *Xlim-1* is expressed at the gastrula stage (Taira et al., 1992), and is required downstream of Sia for organizer formation (Kodjabachian et al., 2001), suggesting that *Xlim-1* may maintain *Sia* target gene expression. The organizer gene *Xotx2* is also expressed in the gastrula stage (Blitz and Cho, 1995), and overexpression of *Xotx2* expands anterior development (Andreazzoli et al., 1997), suggesting a role in maintaining organizer gene expression or organizer function. Inhibition of *Xotx2* activity results in a loss of anterior tissues, including the cement gland, eyes and pharynx (Isaacs et al., 1999), revealing an important role for *Xotx2* in head development.

While Sia, Mix.1, Xlim-1, and Xotx2 likely interact at target promoters to affect organizer gene expression, it remains unknown how early gastrula expression of genes like *Cer* is initiated. The early expression patterns of Sia/Twn, along with the maternal

stores of FoxH1 and Smad2/3, suggest that these effectors may be involved in the initiation of the expression of these organizer genes. Sia/Twn and FoxH1 expression persists until the late gastrula stage (Blythe et al., 2010; Chen et al., 1996; Laurent et al., 1997), suggesting that other factors, such as Mix.1, Mixer, Milk, Xlim-1, or Xotx2 likely continue to maintain organizer gene expression through later stages. Consistent with this idea, zebrafish mutant for *FoxH1* (*schmalspur*, or *sur*) or *Mixer* (*bonnie and clyde*, or *bon*) have relatively mild phenotypes, characterized by a loss of axial tissue and endodermal gene expression, respectively (Kunwar et al., 2003). However, zebrafish mutant for both *FoxH1* and *Mixer* (MZ*sur;bon*) display a more severe phenotype, characterized by loss of dorsal mesoderm and endoderm (Kunwar et al., 2003). Interestingly, while expression of *gsc* expression is reduced in *sur* mutants, and is normal in *bon* mutants, *gsc* expression is completely absent in MZ*sur;bon* mutants (Kunwar et al., 2003), consistent with the idea that FoxH1 may act early in initiating organizer gene expression, while other Nodal effectors act later to maintain gene expression.

1.10. Co-Factor Recruitment in Organizer Formation

While the Wnt and Nodal pathways are essential for organizer gene expression, little is known about co-factor recruitment or chromatin modifications that occur during early embryogenesis. The chromatin of the early embryo is highly acetylated, suggesting that activating chromatin modifications occur during early development (Veenstra, 2002). Inhibition of the activity of p300, a histone acetyltransferase and common co-activator (Ogryzko et al., 1996), results in a loss of mesodermal and organizer gene expression (Kato et al., 1999), suggesting that p300 activity may be required in Wnt and Nodal

mediated gene expression. However, inhibition of p300 activity could be affecting direct targets of the maternal Wnt pathway, as β-catenin has been shown to recruit p300 to target promoters (Hecht et al., 2000). Inhibition of this early Wnt signal would compromise mesodermal and endodermal gene expression, so it remains unclear when during development p300 is acting. p300 activity could be required for zygotic expression of the Wnt effectors Sia and Twn, but not for Sia and Twn function. Or, a more likely scenario is that p300 could be utilized at several time points during organizer formation and embryogenesis, making the exact role of p300 in organizer formation difficult to define. In Nodal signaling, p300 interacts with and acetylates both Smad2 and Smad3 in a ligand dependent manner, enhancing transcription (Inoue et al., 2007; Nishihara et al., 1998; Ross et al., 2006; Tu and Luo, 2007). Similarly, Smad2 recruits the ATP-dependent chromatin modifier Brg1, part of a larger SWI/SNF complex, in a ligand dependent manner (Ross et al., 2006). It should be noted, however, that the interaction of the receptor Smads with p300 have been shown in cell culture systems. Therefore, the role of p300 in mediated a Nodal signal in the early embryo is largely unknown.

Co-factor recruitment to target promoters would likely result in chromatin modifications that could influence temporal and spatial expression of genes. Recent work has found that maternal β-catenin induces methylation of histone H3R8 at the Sia promoter, a mark that primes *Sia* for expression at the MBT (Blythe et al., 2010). Analysis of chromatin marks in gastrula stage *Xenopus tropicalis* embryos revealed that several genes expressed within distinct regions of the embryo, such as the organizer, displayed bivalent chromatin marks, an activating H3K4me3 mark and a repressive H3K27me3 mark (Akkers et al., 2009). However, sequential chromatin

immunoprecipitation revealed that these marks were not on the same regions of DNA, suggesting that regional expression of these genes may be due, in part, to regionally localized chromatin marks within the embryo (Akkers et al., 2009). Similar work in zebrafish revealed that genes may be marked bivalently, with both H3K27me3 and H3K4me3, which is thought to poise genes to respond to signal induction quickly (Vastenhouw et al., 2010). While co-factor recruitment is likely highly important in the function of organizer inducing factors such as Sia or FoxH1, very little is known of co-activator or co-repressor expression in the early embryo. Further work is needed to identify important regulatory domains, chromatin modifications, and co-factors involved in early organzier specification. These modifications and co-factors would likely be important in the regulation of pluripotency or multipotency in the developing embryo, and may also be important in the development and maintenance of somatic stem cells in multiple vertebrate systems.

1.11. Project Approach

In *Xenopus*, formation of the Spemann organizer is essential for the patterning of the embryonic germ layers and vertebrate axis formation. Both the Wnt and Nodal pathways are required for organizer formation, but it remains unclear how these two pathways regulate organizer gene expression in the context of the early embryo. The maternal Wnt pathway activates expression of Sia and Twn, zygotic effectors of the pathway that directly activate expression of the organizer genes *Gsc* and *Cer*. The Nodal pathway is active at MBT, and it signals through maternally deposited stores of the transcription factor FoxH1 and the effectors Smad2 and Smad3. Much evidence suggests that inputs from both the Wnt and Nodal pathways are involved in organizer

gene expression, but little is known about how this occurs. While signaling from Nodal is required for Sia-mediated expression of several organizer genes, it remains unclear whether these pathways are cooperating in the expression of organizer genes. To better understand how the Wnt and Nodal pathways cooperate in organizer formation, we chose to address the following questions:

1.11.1. How do Siamois and Twin activate expression of the target gene Goosecoid?

The *Gsc* promoter contains a Wnt responsive PE that mediates its expression. Work by our lab and others has suggested that Sia and Twn mediate the response to maternal Wnt signals by acting as zygotic effectors of the Wnt signaling pathway. To determine if Sia and Twn directly regulate *Gsc* expression via the Wnt-responsive PE, we mapped the Sia/Twn binding site within the *Gsc* promoter. Sia/Twn bind to a highly conserved element within the *Gsc* PE that is essential for Sia/Twn binding to and activating transcription from the *Gsc* promoter. Sia and Twn are able to dimerize on the PE, forming both homodimers and heterodimers with equivalent function. And finally, we find that Sia/Twn occupy the *Gsc* promoter as both homodimers and heterodimers *in vivo*, suggesting that Sia and Twn may function redundantly.

1.11.2. What is the role of Sia/Twn in organizer formation?

Several lines of evidence suggest that Sia and Twn function is essential for organizer formation. Overexpression of Sia or Twn induces formation of a complete ectopic axis, suggesting that Sia and Twn are sufficient to induce formation of a fully functional organizer. However, recent work claimed that Sia/Twn are only essential for anterior development, implying that trunk organizer function develops independently of Sia/Twn.

To determine the role of Sia and Twn in organizer formation, we used morpholino oligonucleotides to knock down Sia/Twn function in the embryo. Sia/Twn together, but not individually, are required for organizer gene expression and full axis formation, which is consistent with a redundant role for Sia and Twn in organizer formation. In contrast to previous work, Sia/Twn together are required for all the known functions of the organizer, suggesting that Sia/Twn are essential in establishing the organizer domain.

1.11.3. How do Sia/Twn cooperate with Nodal to affect organizer gene expression?

While both Sia/Twn and Nodal are required for organizer formation and organizer gene expression, how these signals are integrated is largely unknown. The *Gsc* promoter contains a Wnt responsive PE and a Nodal responsive DE, suggesting that signals may be integrated at the level of target promoters. However, the mechanism of such integration is not known. Indeed, we find that Sia/Twn and Nodal cooperate to synergistically activate expression of three organizer genes: *Gsc*, *Cer*, and *Chd*. Sia/Twn and Nodal pathway effectors FoxH1 and Smad2/3 occupy endogenous promoters of these genes, and effector occupancy is enhanced when both Sia/Twn and Nodal pathway effectors are present at the promoters. The histone acetyltransferase p300 is recruited to target promoters in response to Sia/Twn or Nodal, suggesting that co-factor recruitment may contribute to transcriptional synergy. Altogether, we identify a common mechanism for regulation of organizer gene expression. This transcription complex likely contributes to the spatial and temporal expression of the organizer genes to ultimately form the organizer.

1.11.4. What are the important domains of Sia/Twn?

Sia and Twn are homeodomain proteins that share a high homology within the N-terminal region, but lack other recognizable domains. The organizer is required for axial development in multiple species, suggesting that transcription factors that regulate organizer gene expression, and thus function similarly to Sia/Twn, may exist in other vertebrates. Similarly, the high level of conservation in the promoter region of the organizer gene *Gsc* suggests that homeodomain factors may regulate its expression in other species. However, to date, vertebrate orthologs of Sia/Twn have not been identified. Elucidation of the regulatory domains of Sia/Twn may help identify proteins with similar function in other vertebrate species. In order to define the regulatory domains of Sia/Twn, we performed structure/function analysis of the similar N-terminal domains of Sia and Twn. The activation domain of Sia lies within the B domain of the protein, while Twn contains two transactivation domains, one each within the A and B domains. We identify a critical amino acid within the Sia A domain which, when mutated, is able to confer transactivation activity to this otherwise inactive domain. And lastly, we explore the conserved lysine residues within the Sia/Twn C domains that may modulate protein activity.

Chapter 2 Siamois and Twin are redundant and essential in formation of the Spemann organizer

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2.1. Summary

The Spemann organizer is an essential signaling center in *Xenopus* germ layer patterning and axis formation. Organizer formation occurs in dorsal blastomeres receiving both maternal Wnt and zygotic Nodal signals. In response to stabilized βcatenin, dorsal blastomeres express the closely related transcriptional activators, Siamois (Sia) and Twin (Twn), members of the paired homeobox family. Sia and Twn induce organizer formation and expression of organizer-specific genes, including *Goosecoid* (*Gsc*). In spite of the similarity of Sia and Twn sequence and expression pattern, it is unclear whether these factors function equivalently in promoter binding and subsequent transcriptional activation, or if Sia and Twn are required for all aspects of organizer function. Here we report that Sia and Twn activate *Gsc* transcription by directly binding to a conserved P3 site within the Wnt-responsive proximal element of the *Gsc* promoter. Sia and Twn form homodimers and heterodimers by direct homeodomain interaction and dimer forms are indistinguishable in both DNA-binding and activation function. Sequential chromatin immunoprecipitation reveals that the endogenous *Gsc* promoter can be occupied by either Sia or Twn homodimers or Sia-Twn heterodimers. Knockdown of Sia and Twn together, but not individually, results in a failure of organizer gene expression and a disruption of axis formation, consistent with a redundant role for

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Sia and Twn in organizer formation. Furthermore, simultaneous knockdown of Sia and Twn blocks axis induction in response to ectopic Wnt signaling, demonstrating an essential role for Sia and Twn in mediating the transcriptional response to the maternal Wnt pathway. The results demonstrate the functional redundancy of Sia and Twn and their essential role in direct transcriptional responses necessary for Spemann organizer formation.

2.2. Introduction

Vertebrate axial development is dependent on the correct formation and function of the dorsal signaling center known as the Spemann organizer (reviewed in Harland and Gerhart, 1997). Spemann organizer function is essential for the dorsoventral and anteroposterior patterning of the embryonic germ layers that serves as a foundation for subsequent axial development (reviewed in Harland and Gerhart, 1997). The organizer is a source of multiple negative regulatory factors, including the secreted antagonists Cerberus, Chordin, and Noggin, and transcriptional repressors such as Goosecoid (Gsc), which act to silence or moderate the activity of TGFβ and Wnt signals within the organizer and adjacent domains (reviewed in De Robertis, 2006). The combined action of these antagonists and repressors establishes signaling gradients and boundaries that confer spatial pattern in the gastrula and organize the embryonic axes during gastrulation (reviewed in De Robertis, 2006).

The organizer forms in response to the combined action of two distinct signaling inputs, the Wnt and Nodal signaling pathways (Harland and Gerhart, 1997). Shortly after fertilization, dorsal determinants localized to the vegetal hemisphere of the embryo are translocated, in a microtubule dependent manner, to the future dorsal side of the embryo

(Heasman, 2006). These dorsal determinants likely include components of the Wnt signaling pathway, such as Wnt11 and LRP6, leading to localized stabilization of βcatenin in a dorsal domain (Kofron et al., 2007; Tao et al., 2005). The maternal Wnt pathway directly activates transcription of Siamois (Sia) and Twin (Twn), closely related paired-type homeodomain proteins, which function as transcriptional activators and zygotic effectors of maternal Wnt signaling (Brannon et al., 1997; Brannon and Kimelman, 1996; Carnac et al., 1996; Crease et al., 1998; Fan et al., 1998; Kodjabachian and Lemaire, 2004; Laurent et al., 1997; Nelson and Gumbiner, 1998; Nishita et al., 2000).

Sia and Twn were identified in functional screens for factors capable of mimicking the developmental activity of the Spemann organizer (Kodjabachian and Lemaire, 2004; Laurent et al., 1997; Lemaire et al., 1995). Targeted ventral expression of Sia or Twn induces ectopic organizer gene expression, as well as the formation of a complete secondary axis consisting of head, trunk and tail tissues (Laurent et al., 1997; Lemaire et al., 1995). The expression profiles of Sia and Twn are identical, both temporally and spatially, and the onset of expression in dorsal blastomeres at the mid-blastula transition, just prior to the initiation of organizer gene expression, is consistent with a significant role for Sia and Twn in activating organizer gene transcription (Laurent et al., 1997; Lemaire et al., 1995). With near identity within the paired-type homeodomains, mediating DNA-binding and target selection, Sia and Twn likely share the same targets for transcriptional activation (Laurent et al., 1997). Given these similarities in expression and DNA-binding domains, it was suggested that Sia and Twn may function as redundant or cooperative regulatory factors in activation of organizer gene expression (Laurent et al., 1997).

Expression of a dominant repressive form of Sia, a fusion of the Engrailed repressor domain with the Sia homeodomain (Eng-Sia), in the dorsal domain of the gastrula results in a complete suppression of organizer gene expression and axis formation, demonstrating that Sia and/or Sia-related proteins are essential for organizer formation (Fan and Sokol, 1997; Kessler, 1997). However, recent knockdown analysis suggests that Sia and Twn are necessary only for anterior axial development (Ishibashi et al., 2008). Injection of a mixture of morpholino antisense oligonucleotides specific for Sia and Twn resulted in a loss of head structures, but trunk and tail development was normal (Ishibashi et al., 2008), suggesting that Sia and Twn are required for head organizer function, but not for the full activity of the Spemann organizer. So while the gain-of-function and dominant repressor studies suggest that Sia and Twn confer full organizer activity (head and trunk organizer) (Kodjabachian and Lemaire, 2001; Laurent et al., 1997; Lemaire et al., 1995), the knockdown studies suggest a role limited to anterior development (head organizer) (Ishibashi et al., 2008). These apparent differences could reflect off-target effects resulting from overexpression of Sia, Twn and Eng-Sia. Alternatively, the knockdown phenotype could represent a partial loss-of-function for endogenous Sia and Twn. Given these contrasting results, further analysis is necessary to define the developmental requirement for Sia and Twn in organizer formation and function.

Sia and Twn are likely direct transcriptional regulators of multiple organizer genes. Sia has been shown to cooperate with Xlim1, Xotx2 and Mix.1 in the direct regulation of Cerberus, and both Sia and Twn directly activate *Gsc* (Fan and Sokol, 1997; Kessler, 1997; Laurent et al., 1997; Yamamoto et al., 2003). *Gsc* is expressed specifically within the organizer domain (Blumberg et al., 1991; Cho et al., 1991; De

Robertis, 2004) where it functions as a transcriptional repressor to suppress Wnt and BMP signaling and maintain organizer identity (Sander et al., 2007; Yao and Kessler, 2001). The *Gsc* promoter contains a distal element (DE) responsive to TGFβ signals and a proximal element (PE) responsive to Wnt signals (Watabe et al., 1995). These two response elements are found in close proximity within the *Gsc* promoter and are conserved in all vertebrate *Gsc* genes (Fig. 2.1A). Previous studies have shown that the Wnt-responsive PE is necessary for Sia and Twn-mediated activation of a *Gsc* reporter construct (Fan and Sokol, 1997; Kessler, 1997; Laurent et al., 1997; Yao and Kessler, 2001), and *in vitro* experiments have revealed that Twn binds to a conserved region within the PE (Laurent et al., 1997). The similarities in the structure, expression and function of Sia and Twn suggest that these proteins likely bind the same sequence in the *Gsc* promoter to activate transcription. However, it is unknown whether Sia and Twn contribute equivalently to the activation of *Gsc* expression. Furthermore, it remains unclear whether Sia and Twn function in an entirely redundant manner in organizer formation, and whether these factors are required for the complete function of the Spemann organizer. Further analysis of the regulation of *Gsc* and other organizer genes by Sia and Twn would provide insight to the developmental and transcriptional mechanisms of organizer formation.

We sought to address these questions, first by defining the conserved sequences within the *Gsc* PE that are required for stable binding of Sia and Twn and consequent transcriptional activation of *Gsc*. In protein interaction assays Sia and Twn form both homo- and heterodimers through direct protein-protein interactions, and we found that the different dimer forms are indistinguishable in both DNA-binding and transcriptional activation function. *In vivo*, Sia and Twn can together occupy the endogenous *Gsc*

promoter, consistent with both homo- and heterodimer formation at the *Gsc* promoter. Knockdown of both Sia and Twn together, but not individually, results in a loss of organizer gene expression and a complete disruption of axis formation. Furthermore, we confirm the prediction that Sia and Twn together are required downstream of the Wnt signaling pathway in axis formation. The results demonstrate the functional redundancy of Sia and Twn and their essential role in direct activation of organizer gene expression and regulation of Spemann organizer formation.

2.3. Results

2.3.1 Siamois and Twin Bind Identical Sequences in the Goosecoid Proximal Element

Sia and Twn have each been identified as direct regulators of *Gsc* expression in previous studies (Fan and Sokol, 1997; Kessler, 1997; Laurent et al., 1997). Sia and Twn share high homology, especially within the third helix of the homeodomain, which is predicted to be the region of the DNA binding domain that imparts specific recognition of target DNA sequences (Laurent et al., 1997; Wilson et al., 1995). Previous biochemical studies indicated that Twn binds to the PE of the *Gsc* promoter at a sequence that contains two consensus homeodomain binding half sites (Laurent et al., 1997). As paired-type homeodomain proteins, Sia and Twn are predicted to bind preferentially to P3 sites, consisting of two inverted TAAT motifs separated by 3 base pairs (Wilson et al., 1995). Examination of the *Xenopus Gsc* PE reveals a near perfect consensus P3 site (-129 to -119) with an additional upstream half site (-136 to -133) (Fig. 2.1A). Alignment of *Gsc* promoter sequences of *Xenopus laevis*, human, mouse and zebrafish reveals a striking conservation of the P3 site and the upstream half site (Fig. 2.1A). The presence

of this conserved P3 site within the *Gsc* PE suggests a role for paired-type homeodomain proteins in *Gsc* transcriptional regulation across species. We sought to investigate whether this site plays a role in mediating the transcriptional response to Sia and Twn in *Xenopus*.

To precisely map the region bound by Sia and Twn within the *Gsc* promoter, DNase footprinting was performed. A fragment of the *Gsc* promoter (-226 to +1) (Watabe et al., 1995) was labeled either on the top or bottom strand, incubated with full length Sia protein, Sia homeodomain (HD), Twn HD or a mixture of Sia HD and Twn HD, and subjected to DNase1 digestion to identify the regions bound and protected. A nearly identical region, containing the conserved P3 site and upstream half site, was protected on both the top (-146 to -115) and bottom strands (-145 to -115) (Fig. 2.1B-D). Sia HD, Twn HD or a mixture of Sia HD and Twn HD protected the same area as full-length Sia, suggesting that the homeodomain alone is sufficient to confer specific binding to the *Gsc* promoter (Fig. 2.1B,C). These results are consistent with previous footprinting analysis with the Twn homeodomain, which showed a protection of -114 to -127 within the *Gsc* PE (Laurent et al., 1997). Two minor protected regions (-103 to -93 and -15bp to +1bp) were detected as well (Fig. 2.1 B,C), but these did not contain apparent homeodomain binding sites and may be either non-specific or cryptic homeodomain binding sites. These results demonstrate that Sia and Twn bind to and protect an identical region of the *Gsc* promoter, which includes a conserved P3 site and upstream half site. The near identity of the Sia and Twn homeodomains predicts that Sia and Twn likely share transcriptional targets (Laurent et al., 1997); our results suggest that Sia and Twn regulate *Gsc* transcription by binding to the highly conserved P3 site within the *Gsc* promoter.

Figure 2.1 Siamois and Twin bind an identical conserved region within the *Gsc* proximal element.

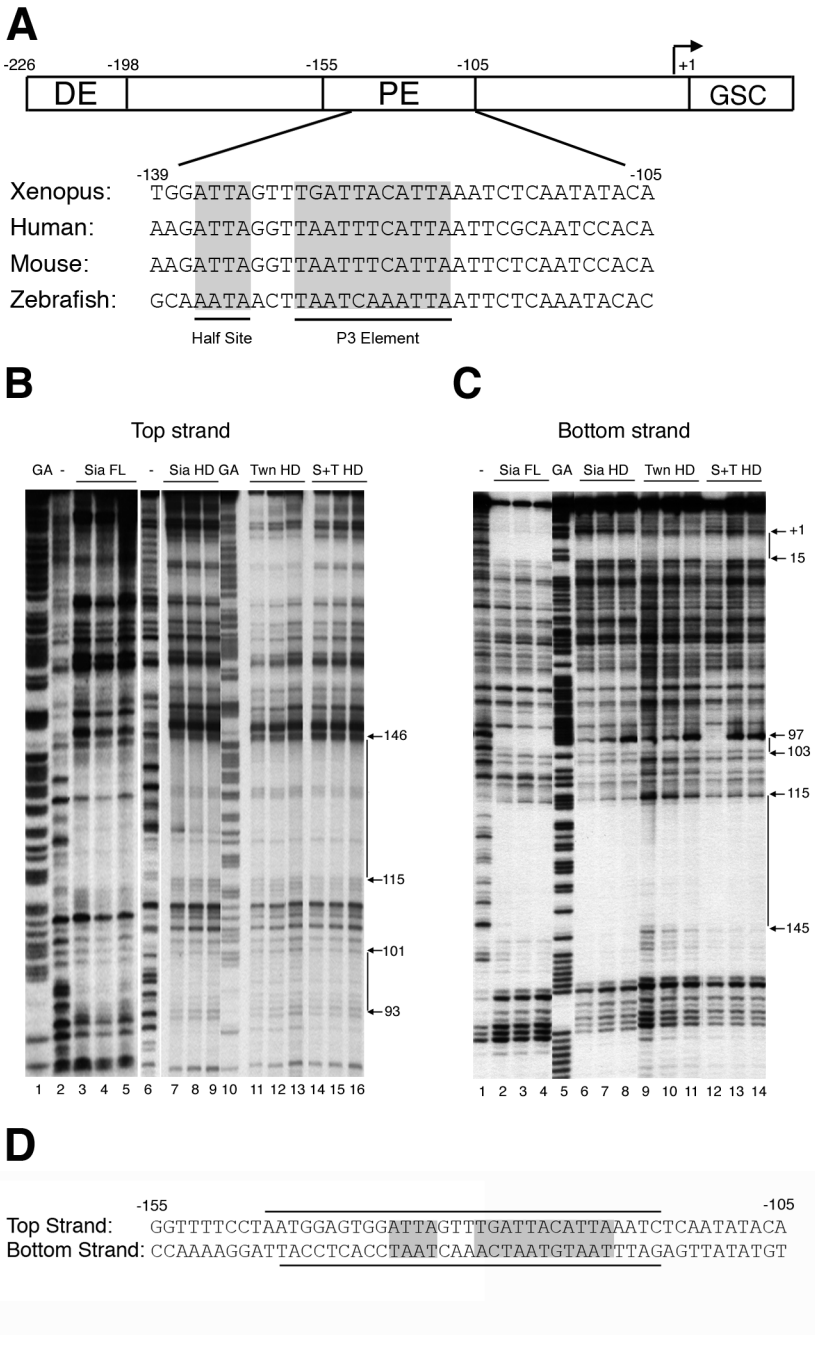


Figure 2.1. Siamois and Twin bind an identical conserved region within the *Gsc* proximal element.

(A) Schematic of the *Gsc* promoter indicating sequence conservation within the Proximal Element (PE) across species. The P3 element and upstream half site are indicated by gray shading. DNase footprinting was performed on the *Gsc* promoter to identify regions protected by Sia and Twn (B,C). A double-stranded fragment of the *Gsc* promoter, radiolabeled on the top (B) or bottom (C) strand, was incubated with full-length Sia, Sia homeodomain (Sia HD), Twn homeodomain, (TwnHD) or a mixture of the Sia and Twn homeodomains (S+T HD). Protected regions are indicated to the right of each autoradiogram (B,C) and summarized in schematic form (D). In addition to the major protected region containing the P3 site and the upstream half site, two minor protected regions (-103 to -93 and -15bp to +1bp) were detected as well, but these did not contain apparent homeodomain binding sites and may be either non-specific or cryptic homeodomain binding sites. The region of protection for the top strand is overlined and for the bottom strand is underlined in (D), with the upstream half site and the P3 site indicated by gray shading. GA indicates a sequencing reaction run with purine terminators, providing a size ladder for DNAse cleavage products

2.3.2 Siamois and Twin Binding to the Goosecoid Promoter is Dependent on Conserved Homeodomain Binding Sites

To determine whether the conserved P3 site and upstream half site are required for Sia binding to the Gsc promoter, electrophoretic mobility shift assays (EMSA) were performed using a double-stranded oligonucleotide probe containing the region protected by Sia and Twn (-146 to -115, referred to as wild-type or WT probe) (Fig. 2.2A). When bound by full-length recombinant Sia protein, the WT probe formed two distinct protein-DNA complexes, a higher mobility complex and a lower mobility complex (Fig. 2.2B). Formation of the higher mobility complex was seen at lower protein concentrations, whereas the lower mobility complex was observed only at higher protein concentrations. Paired-type homeodomain proteins are known to dimerize at higher protein concentration (Wilson et al., 1993), suggesting that the higher mobility complex represents the binding of a Sia monomer to one half site, while the lower mobility complex results from formation of a Sia dimer at the P3 site. Consistent with this idea, palindromic P3 sites have been shown to be occupied by two paired-type homeodomain proteins in a high affinity complex (Wilson et al., 1993), which would suggest that Sia and Twn might both occupy the Gsc promoter to regulate transcription.

To assess the contribution of the upstream half site and P3 site to Sia binding, complex formation was assessed for probes with mutations introduced into the upstream half site (136 MT), the P3 site (127 MT) or both the upstream half site and P3 site (2X MT) (Fig. 2.2A). Sia binding was unaffected by mutation of the upstream half site (136 MT), and both monomer and dimer complexes formed at near identical protein concentrations as observed for the WT probe (Fig. 2.2B). To disrupt the P3 site, one of the half sites comprising the P3 site was mutated (127 MT), and this resulted in a

Figure 2.2 The *Gsc* P3 Element is required for stable binding and transactivation by Sia and Twn.

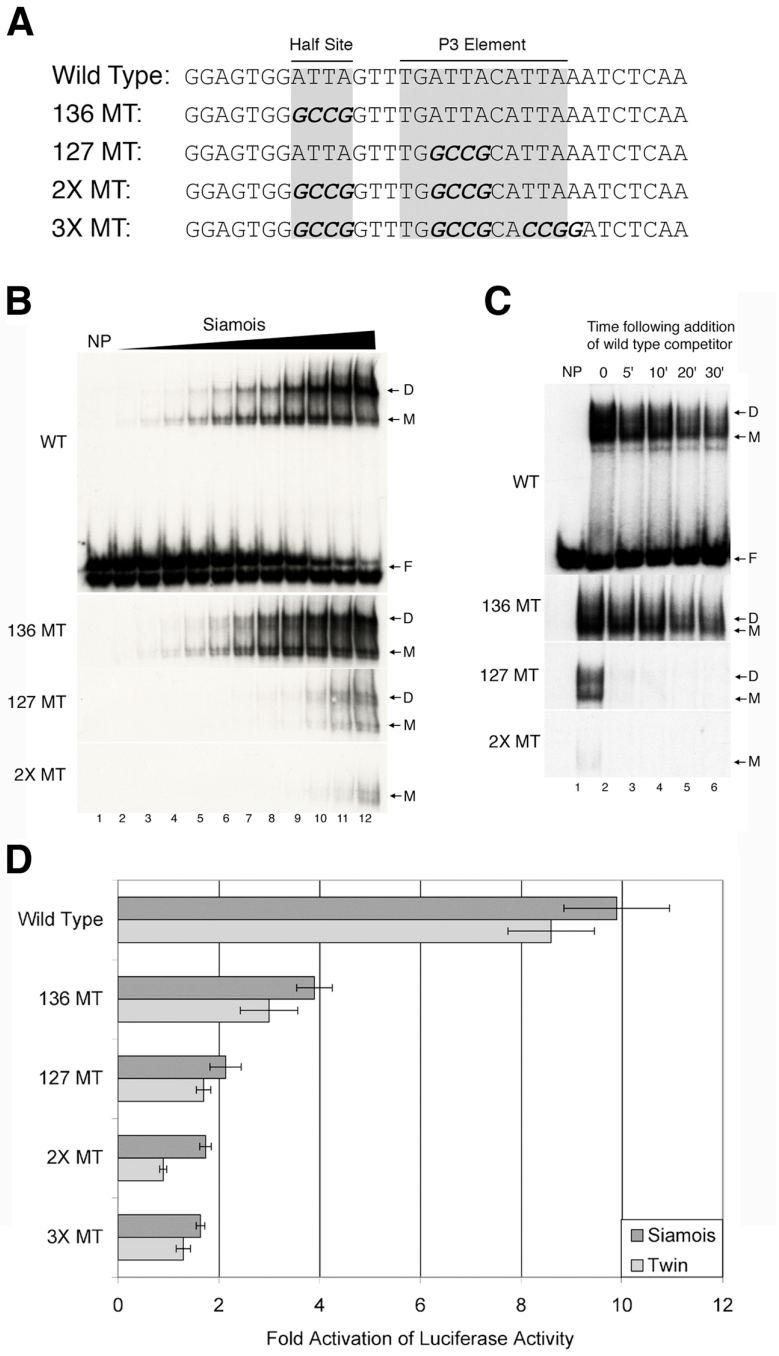


Figure 2.2. The *Gsc* P3 element is required for stable binding and transactivation by Sia and Twn.

(A) Sequence of oligonucleotide probes used in EMSA experiments with the P3 element and upstream half site indicated with gray shading. Mutated nucleotides are indicated with bold italics. (B) Increasing amounts of Sia protein was incubated with the indicated radiolabeled EMSA probes, and predicted monomer (M) and dimer (D) complexes were observed for the WT, 136 MT and 127 MT probes. Only the monomer complex was observed to form on the 2X MT probe and no complex formation was observed on the 3X MT probe (data not shown). (C) Assessment of the stability of the Sia-DNA complex. A constant amount of Sia protein was incubated with the indicated radiolabelled EMSA probe. Following a 20 min preincubation of Sia protein with radiolabelled probe (time 0), an excess of unlabelled WT competitor was added and complex formation was examined at the indicated times (5, 10, 20 and 30 min) following competitor addition. NP, no protein; F, free probe. (D) Requirement for the *Gsc* P3 element and upstream half site in Sia and Twn transactivation. At the one-cell stage Sia or Twn mRNA (100pg) was injected into the animal pole and at the two-cell stage DNA for *Gsc*-Luciferase reporters (100pg) containing the indicated forms of the *Gsc* promoter were injected together with DNA for CMV-Renilla Luciferase (10pg). Animal explants prepared at the blastula stage were assayed for luciferase activity at the midgastrula stage. Values shown are normalized to Renilla luciferase activity, and represent fold activation of basal reporter activity in the absence of injected mRNAs. The mean and standard error for three independent experiments is presented

dramatic reduction of complex formation (Fig. 2.2B). Only at the highest concentrations of Sia protein were monomeric and dimeric complexes detected, but at greatly reduced levels compared to the WT probe (Fig. 2.2B). The continued presence of both the monomer and dimer complexes may reflect low affinity binding of Sia to the two half sites still present in the probe. When both the P3 site and the upstream half site were mutated (2X MT), only a monomeric complex was weakly observed at the highest concentrations of Sia protein (Fig. 2.2B), likely due to Sia binding to the single remaining half site. When all three half sites were mutated (3X MT), no binding of Sia was observed, even at the highest protein concentration (data not shown). Taken together, these results demonstrate that high affinity binding of Sia to the *Gsc* PE is dependent on the conserved P3 site.

To further assess the sequence requirements for stable binding of Sia to the Gsc promoter, EMSA competition assays were performed. Binding of Sia protein to radiolabeled probe (WT, 136 MT, 127 MT or 2X MT) was allowed to reach equilibrium (20 min), a 100-fold molar excess of unlabeled WT probe was then added, and the resulting levels of Sia-DNA complex were assessed at 5, 10, 20 or 30 min after competitor addition (Fig. 2.2C). As expected, Sia binding to the WT probe formed a stable complex with ~50% of the complex still intact 30 min after competitor addition (Fig. 2.2C). Sia binding to a probe mutated for the upstream half site (136 MT) was nearly as stable as WT, while mutation of the P3 site (127 MT) or both sites (2X MT) resulted in an unstable complex that was fully dissociated within 5 min of competitor addition (Fig. 2.2C). The extent of complex dissociation following competitor addition suggests that the P3 site, but not the upstream half site, is essential for stable binding of Sia to the Gsc promoter. To assess whether Sia HD or Twn HD is sufficient for complex

formation at the Gsc promoter, as suggested by the DNase footprinting results (Fig. 2.1), we tested the ability of recombinant Sia HD or Twn HD to bind to the WT and mutant probes (Fig. 2.3). When compared to the results with full-length Sia, no differences in complex formation or sequence requirements were observed for the Sia HD (Fig. 2.3A) or Twn HD (Fig. 2.3B) alone, suggesting that the homeodomain confers the complete binding activity of the full-length protein. In addition, the formation of apparent dimeric protein-DNA complexes by the homeodomains alone suggests that dimer formation for Sia and Twn may be mediated by direct homeodomain interactions. Taken together, these results indicate that Sia and Twn have identical sequence requirements for binding to the Gsc PE, and that the conserved P3 site is required for stable dimeric complex formation.

2.3.3 Siamois and Twin activation of Goosecoid transcription is dependent on conserved homeodomain binding sites

To determine if the conserved homeodomain binding sites required for Sia and Twn complex formation at the *Gsc* promoter are also required for transcriptional activation of the *Gsc* promoter, transcriptional reporters containing either the wild-type *Gsc* promoter (-226 to +1) or the mutated forms described above (Fig. 2.2A) were tested *in vivo*. *Xenopus* embryos were injected at the one-cell stage with either Sia or Twn mRNA, a *Gsc*-luciferase reporter and an internal control renilla reporter (Fig. 2.2D). As expected, Sia or Twn strongly activates the wild-type *Gsc* promoter (~10-fold activation) and no significant difference between Sia and Twn transcriptional activity was observed (Fig. 2.2D). However, mutation of the upstream half site (136 MT) caused an ~60% reduction in reporter activity in response to both Sia and Twn, suggesting that this half

Figure 2.3 The Siamois or Twin homeodomain is sufficient for DNA-binding and complex formation at the *Gsc* proximal element.

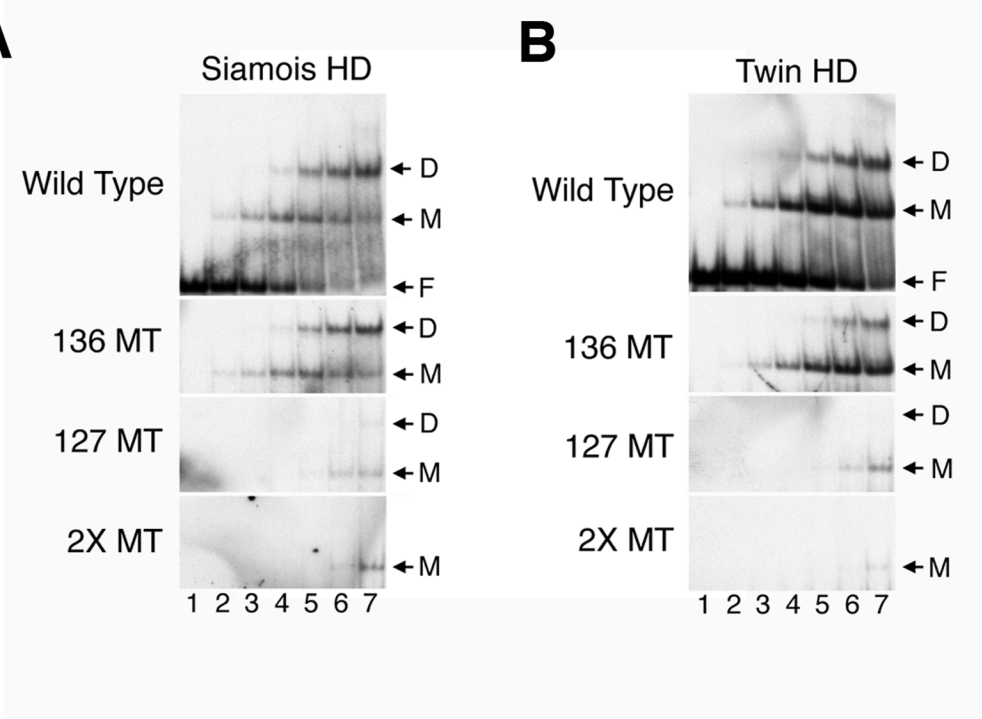


Figure 2.3 The Siamois or Twin homeodomain is sufficient for DNA-binding and complex formation at the *Gsc* proximal element.

Increasing amounts of purified Sia homeodomain (A) or Twn homeodomain (B) was incubated with the indicated radiolabeled EMSA probes. Probe sequences are for wild-type and mutated forms of the *Gsc* proximal element are shown in Fig. 2.2A. Monomer (M) and dimer (D) complexes were observed for the WT, 136 MT and 127 MT probes. The monomer complex only was observed for the 2X MT probe and no complex formation was observed for 3X MT (data not shown). F, free probe.

site, which has a marginal effect on Sia and Twn complex formation on the PE, is required for maximal activity of the Gsc promoter in this assay (Fig. 2.2D). While this site might not contribute to complex formation in vitro, it does seem to contribute to transcriptional activity, perhaps by providing additional contacts for Sia and Twn, or by providing the proper DNA conformation for complex maintenance or cofactor recruitment. Mutation of the P3 site resulted in a near complete loss of transcriptional response (~2-fold activation), while mutation of two (2X MT) or all three half sites (3X MT) fully blocked the response to Sia and Twn (Fig. 2.2D). These results confirm the functional importance of the P3 site in mediating the transcriptional response of Gsc to Sia or Twn, but also reveal a role for the upstream half site in promoting maximal transcriptional response. Given the striking conservation of these homeodomain binding sites in other vertebrate Gsc genes, it is likely that this region of the Gsc promoter is essential for Gsc regulation in other species.

2.3.4 Siamois and Twin Form Homodimers and Heterodimers that Occupy the Goosecoid Promoter

The ability of paired-type homeodomain proteins to dimerize (reviewed in White, 1994), the similar expression and structure of Sia and Twn (Laurent et al., 1997), and the apparent formation of Sia and Twn dimer complexes in DNA-binding assays (Figs. 2.2 and 2.3), suggested that Sia and Twn may form homodimer or heterodimer complexes in regulating *Gsc* transcription. As an initial assessment of the ability of Sia and Twn to form heterodimers, DNA-protein complexes were examined by EMSA using a mixture of recombinant Sia HD and Twn HD (Fig. 2.4A). Since the Sia and Twn HDs are nearly identical in length, a fragment of Sia encompassing the HD and flanking

Figure 2.4 Siamois and Twin form homodimers and heterodimers through direct protein-protein Interactions.

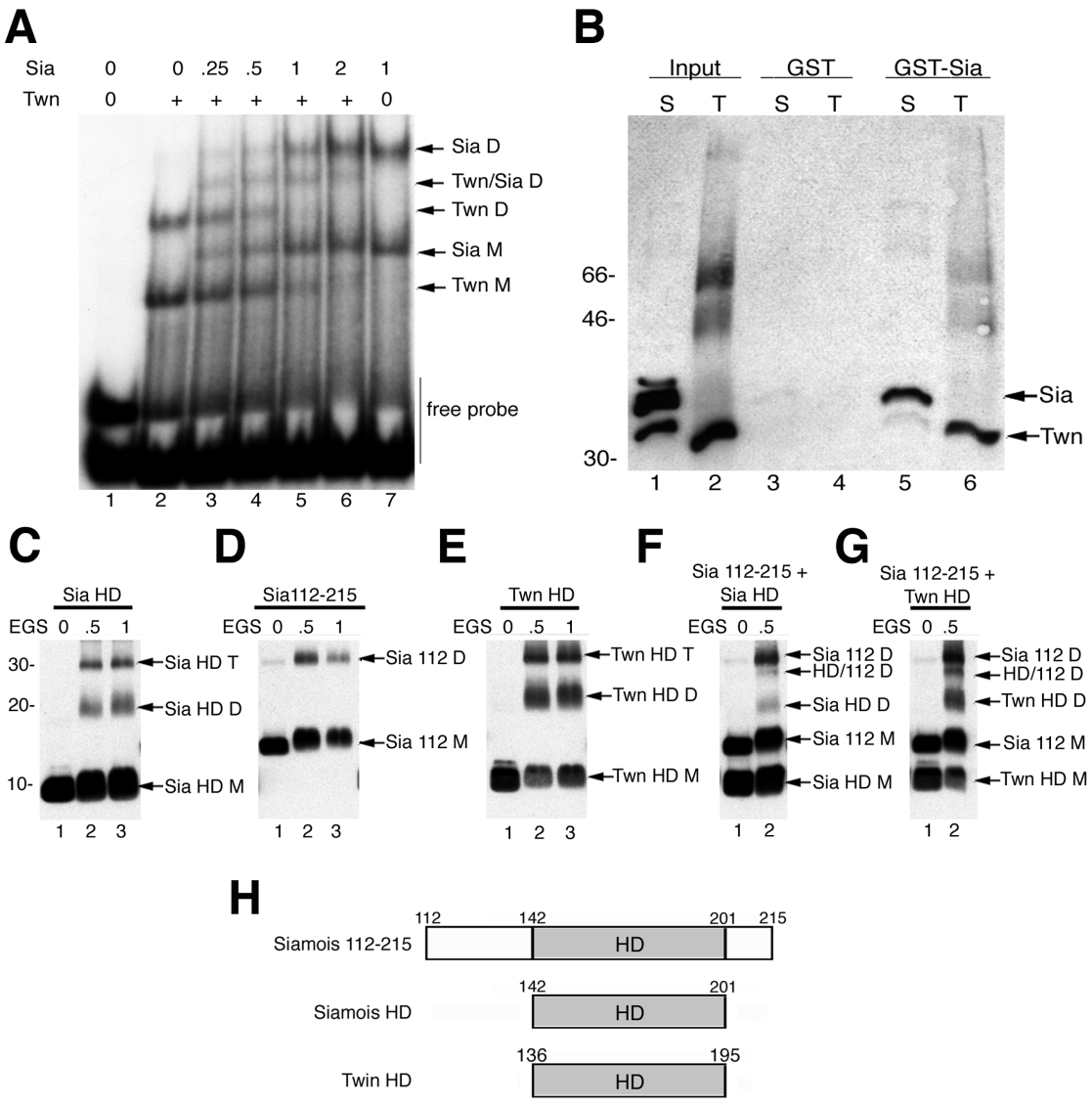


Figure 2.4 Siamois and Twin form homodimers and heterodimers through direct protein-protein Interactions.

(A) EMSA analysis of complex formation for Sia112-215 (lane 7), Twn HD (lane 2) or a combination of both proteins (lanes 3-6) bound to the WT Gsc probe. Twn HD concentration was constant (1 mM), while increasing concentrations of Sia112-215 were combined with Twn HD, as indicated at top in mM. Predicted complex formation indicated on right (M, monomer; D, dimer). (B) Protein interactions of purified Sia and Twn. GST pulldown analysis using purified full-length GST-Sia, His-Sia and His-Twn. Western blot analysis for His-tagged proteins indicating input proteins (lanes 1-2), lack of protein recovery with GST alone (lanes 3-4), and recovery of both His-Sia and His-Twn with GST-Sia (lanes 5-6). Protein size markers are indicated to the left in kD. (C-G) Crosslinking analysis of Sia and Twn dimerization. Homodimeric complex formation shown for Sia HD (C), Sia112-215 (D), Twn HD (E), and Sia112-215 and Sia HD (F), and heterodimeric complex formation for Sia 112-215 and Twn HD (G). Predicted complex formation is indicated on the right (M, monomer; D, dimer; T, trimer), and protein size markers are indicated on the left in kD. Concentration of EGS (Ethylene Glycol-bis (succinic acid N-hydroxysuccinimide ester)) protein crosslinker (mM) is indicated at top. (H) Diagram of the Sia and Twn protein fragments used for the EMSA (A) and crosslinking (C-G) analyses

sequence (Sia112-215) was used to distinguish it from the Twn HD (136-195) based on mobility (diagrammed in Fig. 2.4H). Sia112-215 alone or Twn HD alone each formed two distinct complexes when bound to the WT probe (Fig. 2.4A, lanes 2 and 7), and these correspond to predicted monomer and dimer complexes observed in the studies above (Figs. 2.2 and 2.3). When Sia112-215 and Twn HD were combined in the DNA-binding assay, an additional complex formed that was intermediate in size to the Sia112-215 homodimer and the Twn HD homodimer, consistent with the formation of a Sia-Twn heterodimer (Fig. 2.4A, lanes 3-6). The results suggest that Sia and Twn can form both homodimers and heterodimers on the *Gsc* PE. These dimer forms are likely a result of direct protein-protein interactions, as purified His-Sia and His-Twn binds to a purified GST-Sia fusion protein (Fig. 2.4B, lanes 5-6), but not to GST alone (Fig. 2.4B, lanes 3-4). Therefore, direct and stable protein interaction, in the absence of a DNA-binding site, mediates the formation of Sia homodimers and Sia-Twn heterodimers, and the homodimers and heterodimers appear to form at equal efficiency.

The DNA-binding analyses described above (Figs. 2.2B,C, 2.3, 2.4) suggest that the homeodomain alone is sufficient for homo-and heterodimerization of Sia and TwnTo determine if the homeodomain alone is sufficient for dimerization in the absence of DNA, recombinant His-Sia and His-Twn proteins (Fig. 2.4H) were combined and crosslinked to stabilize protein complexes (Fig. 2.4C-G). The Sia HD (142-201), a larger fragment of Sia (112-215), and the Twn HD (136-195) each formed homodimers, as well as higher molecular weight complexes (Fig. 2.4C-E). When Sia112-215 was combined with either the Sia HD or Twn HD, intermediate sized complexes were formed that demonstrate the formation of a Sia homodimer and a Sia-Twn heterodimer (Fig. 2.4F,G). Taken together, these observations indicate that Sia and Twn homodimers and heterodimers can form by

direct protein interactions of the homeodomain in the absence of a DNA-binding site. These results are consistent with previous structural predictions of paired type homeodomain proteins suggesting that the homeodomain can mediate both protein-protein interactions as well as DNA-protein interactions (Wilson et al., 1995). Furthermore, the results strongly predict that Sia and Twn homodimers, as well as Sia-Twn heterodimers occupy the *Gsc* PE to activate transcription.

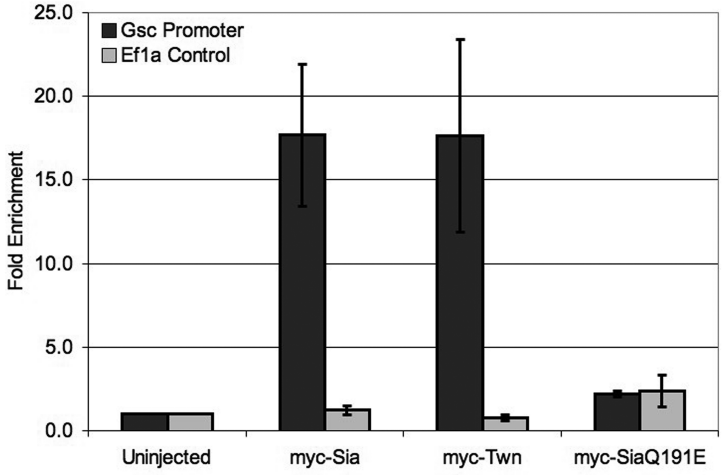
To assess the occupancy of the endogenous *Gsc* promoter by Sia and Twn, chromatin immunoprecipitation (ChIP) in whole embryos was performed (Blythe et al., 2009). Myc-tagged Sia or myc-tagged Twn were immunoprecipitated using an anti-myc antibody and quantitative PCR was performed for either the endogenous *Gsc* promoter or for the *Ef1*α genomic locus as control (Fig. 2.5A). Both Sia and Twn bound robustly and specifically to the *Gsc* promoter (~18-fold enrichment over background) (Fig. 2.5A). This occupancy is dependent on the DNA-binding function of the homeodomain, as an inactivating point mutation (SiaQ191E) in the critical third helix of the homeodomain (Kessler, 1997) impairs occupancy of the *Gsc* promoter (Fig. 2.5A). As predicted, these data indicate that Sia and Twn occupy the endogenous *Gsc* promoter, and that this occupancy is dependent on a functional homeodomain.

While the standard ChIP analysis demonstrates that Sia and Twn occupy the endogenous *Gsc* promoter, it cannot determine whether Sia and Twn occupy the *Gsc*

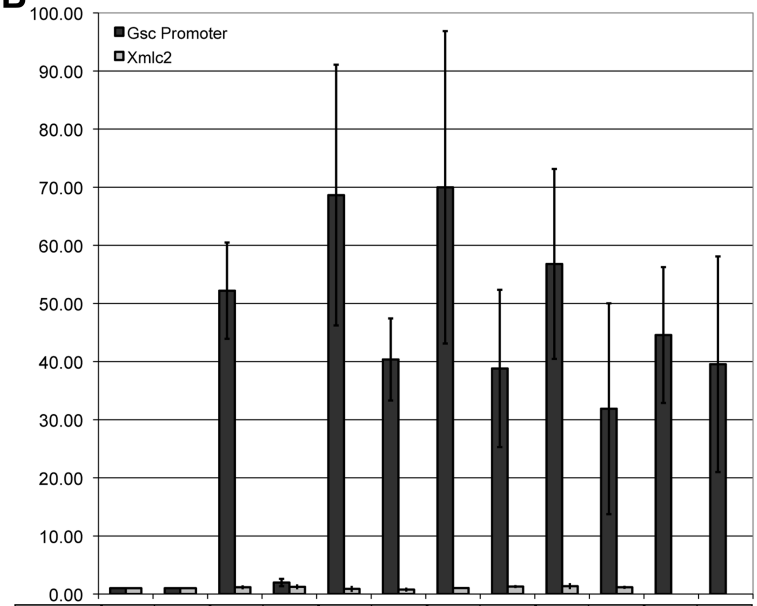
promoter at the same time, which is predicted for Sia-Twn heterodimer formation *in vivo*. To assess the occupancy of the *Gsc* promoter by Sia and Twn homodimers and heterodimers, sequential ChIP was performed in gastrula stage embryos. Differentially tagged forms of Sia or Twn were coexpressed and sequential immunoprecipitations were carried out for each eptiope-tagged form to define the composition of the protein

Figure 2.5 Siamois and Twin homodimers and heterodimers occupy the endogenous *Gsc* promoter.

A



B



Myc-Sia			+	+	+	+					+	+
Myc-Twn							+	+	+	+		
GST			+	+								
GST-Sia					+	+	+	+				
GST-Twn									+	+	+	+
1st IP	myc	myc	myc	myc	myc	myc	myc	myc	myc	myc	myc	myc
2nd IP		GST		GST		GST		GST		GST		GST

Figure 2.5 Siamois and Twin homodimers and heterodimers occupy the endogenous *Gsc* promoter.

(A) Genomic regions recovered by chromatin immunoprecipitation for myc-Sia, myc-Twn or myc-SiaQ191E were evaluated by quantitative PCR (QPCR) for either the *Gsc* promoter or *EF1*α locus as control. The mean fold enrichment (normalized to uninjected samples) and standard error for three independent experiments in presented. (B) Genomic regions recovered by sequential chromatin immunoprecipitation were evaluated by QPCR for the *Gsc* promoter or *Xmlc2* locus as control. Differentially tagged forms of Sia and Twn were coexpressed, samples were subjected to two rounds of immunoprecipitation, and recovered genomic sequences were analyzed by QPCR for each round. Coinjected mRNAs are indicated for myc-Sia, myc-Twn, GST-Sia and GST-Twn, and the order of the myc and GST immunoprecipitations are indicated as 1st IP and 2nd IP. As a control, a first immunoprecipitation with myc-Sia and a second immunoprecipitation with GST alone was also performed. Neither the *Gsc* nor *Xmlc* genomic regions were significantly recovered from the second immunoprecipitation.The mean fold enrichment (normalized to uninjected samples) and standard error for five independent experiments in presented.

complex bound at the *Gsc* promoter. Western blot analysis confirmed equivalent levels of Sia and Twn expression in these studies (data not shown). Genomic DNA recovered in each round of immunoprecipitation was analyzed by QPCR for the *Gsc* promoter and the *Xmlc2* genomic region as control (Fig. 2.5B). The sequential ChIP results are consistent with formation of both Sia-Sia homodimers and Twn-Twn homodimers. The *Gsc* promoter was highly enriched in sequential ChIP for either myc-Sia and GST-Sia or myc-Twn and GST-Twn, while *Xmlc2* genomic sequences were not recovered (Fig. 2.5B). As additional controls, if GST alone was coexpressed with myc-Sia, the *Gsc* promoter was not recovered in GST-containing complexes (Fig. 2.5B). These sequential ChIP studies demonstrate that Sia and Twn homodimers can occupy the endogenous *Gsc* promoter.

Finally, to assess *Gsc* occupancy by Sia-Twn heterodimers, myc-Twn and GST-Sia were coexpressed and subjected to sequential ChIP. The *Gsc* promoter was robustly recovered in both rounds of immunoprecipitation (~60-fold and ~30-fold for myc-Twn and GST-Sia, respectively), consistent with occupancy of the *Gsc* promoter by Sia-Twn heterodimers (Fig. 2.5B). A similar result was obtained when coexpressing myc-Sia and GST-Twn (~50-fold and ~40-fold, respectively), further supporting the conclusion that Sia-Twn heterodimers occupy the endogenous *Gsc* promoter (Fig. 2.5B). Consistent with direct protein-protein interactions in dimer formation at the *Gsc* promoter, sequential ChIP of myc-Sia or myc-Twn with GST-SiaQ191E, a DNA-binding inactive mutant, also results in recovery of the *Gsc* promoter (data not shown). This suggests that SiaQ191E interacts directly with wild-type Sia or Twn at the *Gsc* promoter. Taken together, the ChIP data confirm that *Gsc* is a direct target of Sia and Twn, and that these factors are capable of occupying the endogenous promoter as homodimers or heterodimers.

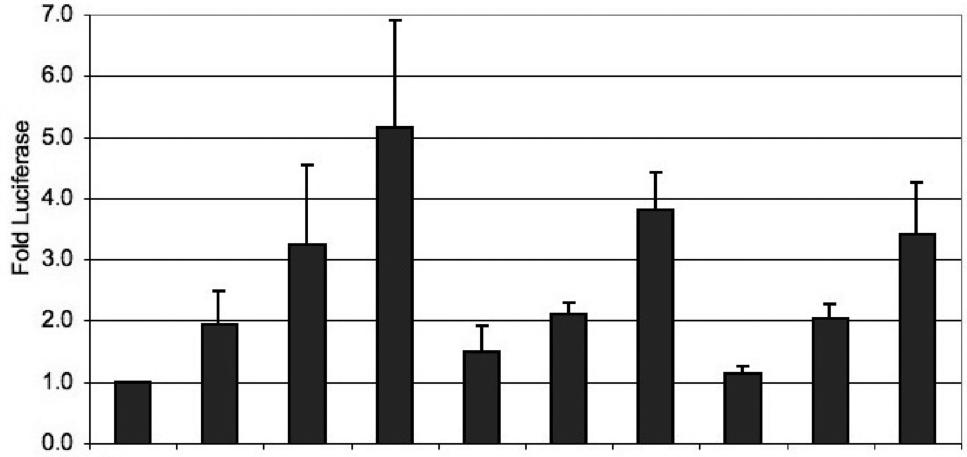
2.3.5 Siamois and Twin Homodimers and Heterodimers have Similar Transcriptional and Developmental Function

The expression, DNA-binding, protein interaction, transcriptional and developmental analyses of Sia and Twn, presented both here and in previous studies (Kodjabachian and Lemaire, 2001; Laurent et al., 1997; Lemaire et al., 1995), suggest that Sia and Twn function equivalently within the context of all available studies. However, our demonstration that Sia-Twn heterodimers form and can occupy the endogenous *Gsc* promoter raises the possibility that the heterodimer complex has distinct function, and may differ from the homodimer forms in either transcriptional or developmental function. To assess the transcriptional and developmental function of Sia-Twn heterodimers, dose response analysis was performed for Sia alone, Twn alone, or the combination of Sia and Twn in a luciferase reporter assay and in an ectopic axis induction assay. The transcriptional response of the WT *Gsc*-luciferase reporter to increasing doses of Sia alone or Twn alone (3, 10 or 30pg mRNA) were similar, with maximal responses of ~5-fold for Sia and ~4-fold for Twn (Fig. 2.6A). When Sia and Twn mRNAs were combined and injected at a total dosage equal to that used for the individual factors (1.5+1.5, 5+5, or 15+15pg), a similar transcriptional dose response was observed (~3.5-fold maximal response) (Fig. 2.6A). These results suggest that Sia and Twn homodimers and heterodimers have similar transactivation function.

Sia and Twn were originally identified based on their ability to mimic the axis-inducing activity of the Spemann organizer when ectopically expressed in ventral blastomeres of the *Xenopus* embryo (Laurent et al., 1997; Lemaire et al., 1995). To assess the developmental function of Sia and Twn homodimers and heterodimers, a single ventral blastomere was injected at the four-cell stage with Sia alone, Twn alone or

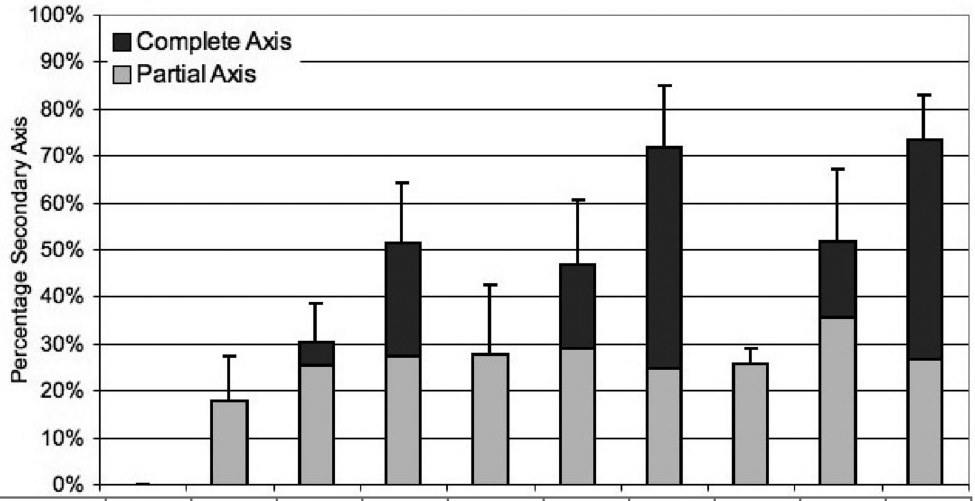
Figure 2.6 Siamois and Twin homodimers and heterodimers have indistinguishable transactivation and axis induction function *in vivo*.

A



Sia (pg)		3	10	30				1.5	5	15
Twn (pg)					3	10	30	1.5	5	15

B



Sia (pg)		1	3	10				0.5	1.5	5
Twn (pg)					1	3	10	0.5	1.5	5
n=	112	56	99	84	54	90	85	47	87	90

Figure 2.6 Siamois and Twin homodimers and heterodimers have indistinguishable transactivation and axis induction function *in vivo*.

(A) At the one-cell stage the animal pole was injected with Sia, Twn or a mixture of both mRNAs at the indicated doses and at the two-cell stage DNA for WT *Gsc*-Luciferase reporter (100pg) was injected together with DNA for CMV-Renilla Luciferase (10pg). Animal explants prepared at the blastula stage were assayed for luciferase activity at the midgastrula stage. Values shown are normalized to Renilla luciferase activity, and represent fold activation of basal reporter activity in the absence of injected mRNAs. The mean increase in luciferase activity and standard error for five independent experiments is presented. (B) At the 4-cell stage a single ventral blastomere was injected with Sia, Twn or a mixture of both mRNAs at the indicated doses. Embryos were scored for ectopic axis induction at the neurula stage. The partial axis class contained ectopic trunk structures extending anterior to the otic vesicle. The complete axis class contained trunk and head structures, including eyes and cement gland. The mean percentage and standard error for five independent experiments is presented. n, total embryos analyzed for each experimental condition.

a combination of Sia and Twn. At low dosage (1pg) Sia or Twn induced partial ectopic axes consisting of tail and trunk structures, but lacking head structures (~20% and ~30% for Sia and Twn, respectively) (Fig. 2.6B). At higher dosage (3 and 10pg) complete ectopic axes, including head structures, were observed at increasing frequency (~25% and ~45% for Sia and Twn at 10pg, respectively) (Fig. 2.6B). When Sia and Twn were injected at a combined dosage equal to the individual mRNAs (0.5+0.5, 1.5+1.5, or 5+5pg), a similar response profile for axis induction was observed. At low dose, Sia+Twn induced partial ectopic axes (~25% at 0.5+0.5pg), and with higher dosage an increasing frequency of complete ectopic axes was observed (~15% and ~45% for Sia+Twn at 1.5+1.5 and 5+5pg, respectively) (Fig. 2.6B). Therefore, under conditions where Sia-Twn heterodimers would likely form, no cooperative or synergistic transcriptional activity or induction of axis formation is observed, but rather the response observed is similar to that obtained with equivalent doses of Sia or Twn alone. Taken together, these results indicate that Sia and Twin homodimers and Sia-Twn heterodimers have indistinguishable function *in vivo*, both in their ability to activate transcription and induce axis formation.

2.3.6 Siamois and Twin are redundant and essential for axial development and organizer formation

In previous studies the function of Sia and Twn was disrupted either with a dominant repressive Eng-Sia fusion protein (Fan and Sokol, 1997; Kessler, 1997) or by simultaneous knockdown of Sia and Twn (Ishibashi et al., 2008). In both cases Sia and Twn were found to be essential for organizer formation and axial development, although the disruption of organizer function differs in severity for these two approaches. While

Eng-Sia completely suppressed organizer and axis formation (Fan and Sokol, 1997; Kessler, 1997), the double knockdown resulted in a less severe phenotype, with loss of head, but not trunk or tail structures (Ishibashi et al., 2008). These differences could reflect off-target effects of Eng-Sia or incomplete knockdown of Sia and Twn. Despite this discrepancy in the functional analysis of Sia and Twn, our results strongly predict that Sia and Twn function equivalently and redundantly in organizer formation. To more clearly establish the requirement for Sia and Twn in organizer formation, and to assess their predicted functional redundancy, Sia and Twn were knocked down individually and in combination.

Translation-blocking morpholino oligonucleotides were designed to specifically target Sia or Twn. The specificity and efficacy of these oligonucleotides was assessed in protein translation and axis induction assays (Fig. 2.7). In an *in vitro* translation assay, the Sia-specific morpholino oligonucleotide (MO) blocked translation of Sia, but not Twn. Conversely, the Twn MO blocked Twn translation, but not Sia (Fig. 2.7A). Myc-tagged forms of Sia and Twn, in which a distinct translational start site is used, were insensitive to either Sia MO or Twn MO (Fig. 2.7A). To assess the function blocking activity of the MOs, their ability to inhibit ectopic axis induction by Sia or Twn mRNA was examined. Injection of Sia or Twn mRNA into a single ventral blastomere at the four-cell stage resulted in induction of complete ectopic axes in most embryos (94% and 72% for Sia and Twn, respectively) (Fig. 2.7C,D), and axis induction was greatly reduced in the presence of the corresponding MO (28% and 6% for Sia and Twn, respectively) (Fig. 2.7I,M), but not with the unmatched MO (95% and 73% for Sia and Twn, respectively) (Fig. 2.7J,L). The axis-inducing activity of myc-Sia and myc-Twn was unaffected by either MO (insets Fig. 2.7I,M). Therefore, the Sia MO and Twn MO are effective and

Figure 2.7 Morpholino antisense oligonucleotides specifically block the translation and biological activity of Siamois and Twin.

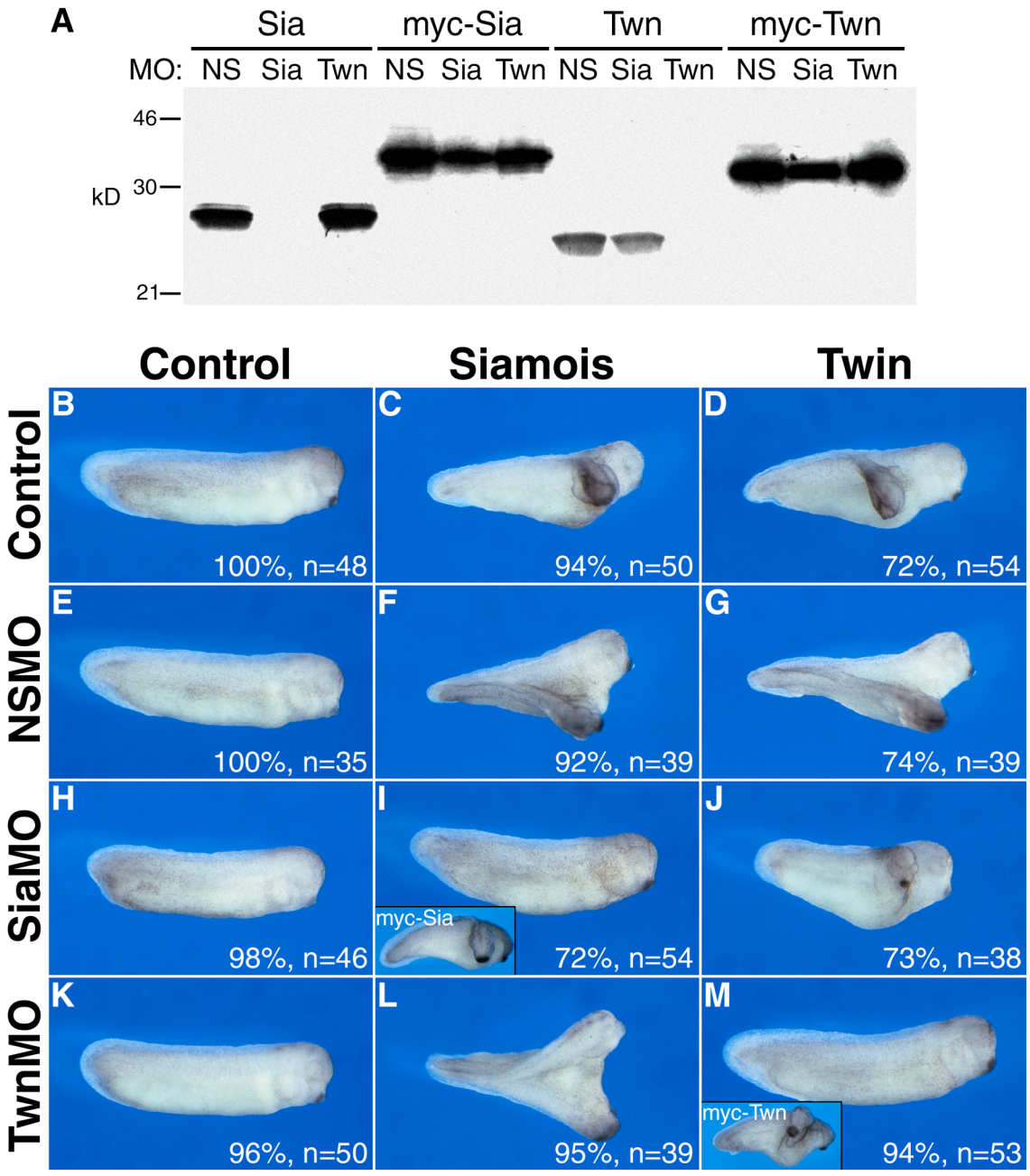


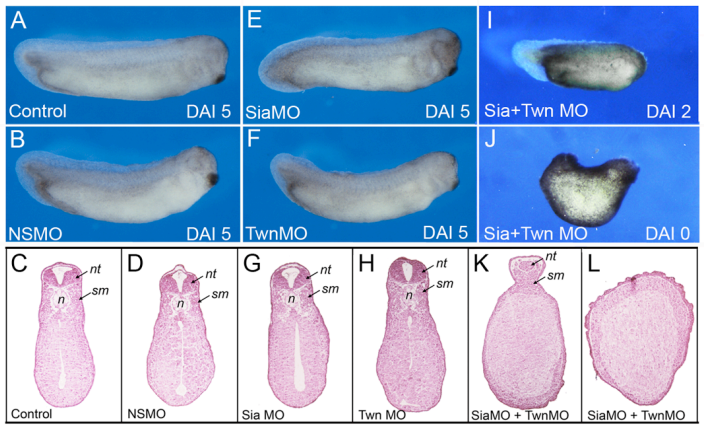
Figure 2.7 Morpholino antisense oligonucleotides specifically block the translation and biological activity of Siamois and Twin.

(A) *In vitro* translation reactions programmed with DNA constructs (1µg) encoding native Sia or Twn, or myc-tagged forms of Sia or Twn, in the presence of oligonucleotides (100ng) specific for Sia or Twn, or a non-specific control oligonucleotide (NS). Translation products were labeled with ^{35}S-methionine, resolved by 12% SDS-PAGE, and visualized by autoradiography. Protein size markers are on the left. The Sia MO blocked translation of Sia, but not Twn. The Twn MO blocked translation of Twn, but not Sia. Neither oligonucleotide blocked translation of myc-Sia or myc-Twn, which have distinct upstream translation start sites. The NSMO oligonucleotide had no translation blocking activity for any of the proteins. (B-M) Inhibition of axis induction by Sia- or Twn-specific oligonucleotides. At the 4-cell stage both ventral blastomeres were injected with (E-G) a non-specific control morpholino oligonucleotide (NSMO, 25ng), (H-J) a Sia-specific oligonucleotide (SiaMO, 25ng), or (K-M) a Twn-specific oligonucleotide (TwnMO, 25ng). At the 8-cell stage a single ventral blastomere was injected with 20pg of (C,F,I,L) Sia, (D,G,J,M) Twn, (I, inset) myc-Sia, or (M, inset) myc-Twn mRNA. The Sia MO blocked axis induction by Sia, but not Twn. The Twn MO blocked axis induction by Twn, but not Sia. myc-Sia and myc-Twn were insensitive to the corresponding oligonucleotides and the NSMO oligonucleotide did not block axis induction for either Sia or Twn. Whole embryo morphology (dorsal up, anterior right) is shown at the tailbud stage, with percentage of embryos displaying the representative phenotype and total embryos analyzed indicated in the lower right for each panel. (B) Uninjected control

specific in blocking the translation and developmental function of Sia and Twn.

To determine the requirement for Sia and Twn in axial development and organizer formation, Sia and Twn were knocked down in the dorsal domain of the embryo, the region of their endogenous expression. At the four-cell stage, both dorsal embryo.blastomeres were injected with the Sia MO or Twn MO individually, or with a combination of both MOs, and axial development was assessed at the tailbud stage (Fig. 2.8A-B,E-F,I-J). Injection of each individual MO, or a control non-specific MO unrelated to Sia or Twn, had little or no effect on axial development (90-100% normal axis formation) (Fig. 2.8A-B,E-F,M). Embryos injected with both Sia MO and Twn MO displayed severe axial defects with loss of head structures, and reduction or loss of trunk and tail structures (Fig. 2.8I-J). Phenotypic severity for the double knockdown embryos ranged from complete ventralization with loss of all axial structures (DAI 0) (Fig. 2.8J) to loss of head with reduction of trunk and tail (DAI 1-2) (Fig. 2.8I), and the majority of injected embryos displayed severe axial defects (90% DAI 0-1 at highest MO dosage) (Fig. 2.8M) (Kao and Elinson, 1989). Histological analysis was performed to examine axial development in the single and double knockdown embryos (Fig. 2.8C-D,G-H,K-L). Axis formation was normal in embryos injected with the Sia MO, Twn MO or the control MO, with notochord, somitic muscle and neural tube formation indistinguishable from uninjected controls (Fig. 2.8C-D,G-H). Double knockdown embryos displayed axial defects ranging from partial ventralization (loss of notochord and fusion of somitic muscle across the midline) (Fig. 2.8I,K) to complete ventralization (loss of notochord, muscle and neural tube) (Fig. 2.8J,L). The severity of axial defects was dependent on the dosage of Sia and Twn MOs. At lower doses (25 ng of each MO), less severe axial defects were observed (loss of head and reduction of trunk and tail; 72% with DAI 2-4),

Figure 2.8 Siamois and Twin function redundantly in axial development and organizer formation.



M

Injection	n=	DAI 5	4	3	2	1	0
Control	103	93%	7%	0%	0%	0%	0%
Nonspecific MO 50ng	55	89%	11%	0%	0%	0%	0%
Sia MO 25ng	39	92%	8%	0%	0%	0%	0%
Twn MO 25ng	43	93%	7%	0%	0%	0%	0%
2.5ng Sia + 2.5ng Twn MO	18	44%	50%	6%	0%	0%	0%
25ng Sia + 25ng Twn MO	39	10%	31%	18%	23%	15%	3%
50ng Sia + 50ng Twn MO	41	0%	0%	10%	0%	41%	49%

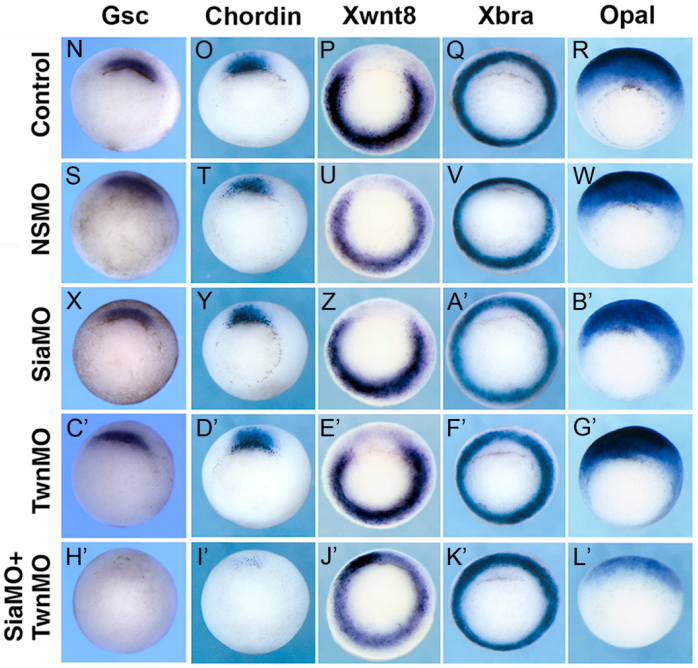


Figure 2.8 Siamois and Twin function redundantly in axial development and organizer formation.

(A-L) At the 4-cell stage both dorsal blastomeres were injected with (B, D) a non-specific control morpholino oligonucleotide (NSMO, 50ng), (E,G) a Sia-specific oligonucleotide (SiaMO, 25ng), (F, H) a Twn-specific oligonucleotide (TwnMO, 25ng), or (I, K) a lower dose combination of the Sia and Twn oligonucleotides (SiaMO+TwnMO, 25ng+25ng) or (J, L) a higher dose combination of Sia and Twn oligonucleotides (SiaMO+TwnMO, 50ng+50ng). (A-B, E-F, I-J) Whole embryo morphology (dorsal up, anterior right) and (C-D, G-H, K-L) transverse histological sections (dorsal up) are shown at the tailbud stage. Dorsoanterior index (DAI) is indicated in the lower right corner for these representative embryos. (M) Quantification of axial defects (DAI scores) observed for Control, NSMO, SiaMO, TwnMO and increasing doses of Sia+TwnMO. Axial structures are indicated for the histological sections (n, notochord; sm, somitic muscle; nt, neural tube). Axial development was normal for embryos injected with the individual control, Sia or Twn MO, while coinjection of Sia and Twn MO resulted in severe axial defects, including loss of head structures, and reduction or loss of trunk and tail structures. Histological samples are presented for two examples of the double knockdown phenotype; (K) a partial loss of axial development with absence of notochord, somitic muscle crossing the midline, and mispatterning of the neural tube, and (L) a complete loss of axial development with no notochord, somitic muscle or neural tube. (N-L') Whole mount in situ hybridization analysis of gene expression at the early gastrula stage (stage 10.25). Embryos injected with 50ng each of NSMO (S-W), SiaMO (X-B'), TwnMO (C'-G') or a combination of SiaMO and TwnMO (50ng + 50ng) (H'-L') were analyzed for organizer expression of *Gsc* (N, S, X, C', H') and *Chordin* (O, T, Y, D', I'), ventrolateral expression of *Xwnt8* (P, U, Z, E', J'), panmesodermal expression of *Xbra* (Q, V, A', F', K'), and neural plate expression of *Opal* (R, W, B', G', L'). Shown are vegetal views with dorsal up (*Gsc*, *Chordin*, *Xwnt8* and *Xbra*) and dorsal-vegetal views with dorsal up (*Opal*). Double knockdown of Sia and Twn together resulted in a reduction or loss of *Gsc* expression in 77% of embryos and a reduction or loss of *Chordin* expression in 100% of embryos.(A,C,N-R) Uninjected control embryos.

while at higher doses of MOs (50 ng of each MO), 90% of embryos displayed a near compete loss of axial structures (DAI 0-1) (Fig. 2.8M). To confirm the specificity of the developmental defects observed, rescue experiments were performed (Fig. 2.9). The severe axial defects observed for the double knockdown (79% axial defects) (Fig. 2.9B) were fully rescued by expression of either myc-Sia or myc-Twn (71% and 76% normal for myc-Sia and myc-Twn, respectively) (Fig. 2.9D,F). These studies demonstrate that Sia and Twn are functionally redundant and together are essential for development of head, trunk and tail structures of the body axis. We note that the severity of the axial defects observed are consistent with the Eng-Sia studies (Fan and Sokol, 1997; Kessler, 1997), but not with the previous knockdown studies (Ishibashi et al., 2008), suggesting that a more complete knockdown reveals a requirement for Sia and Twn in tail, trunk and head development. Consistent with this idea, injection of a mixture Sia MO and Twn MO at lower dosage resulted in reduction of head development with little effect on trunk and tail formation (Fig. 2.8M) similar to the previously reported phenotype (Ishibashi et al., 2008).

To establish the developmental origins of the axial defects resulting from Sia and Twn knockdown, gene expression was examined at the early gastrula stage. Knockdown of Sia or Twn individually had no effect on organizer (*Gsc*, *Chordin*), ventral mesodermal *(Xwnt8*), panmesodermal (*Brachyury*) or neural plate (*Opal*) gene expression (Fig. 2.8X-G'), as was the case for the non-specific control MO (Fig. 2.8S-W). In contrast, simultaneous knockdown of both Sia and Twn resulted in a near complete loss of *Gsc* (77% reduced or absent expression) (Fig. 2.8H') and *Chordin* (100% reduced or absent expression) (Fig. 2.8I'), an expansion of *Xwnt8* into the organizer domain (Fig. 2.8J'), a loss of *Opal* in the neural plate (Fig. 2.8K'), but no change in *Brachyury* expression (Fig.

Figure 2.9 Rescue of axial development in the Siamois-Twin double knockdown embryo.

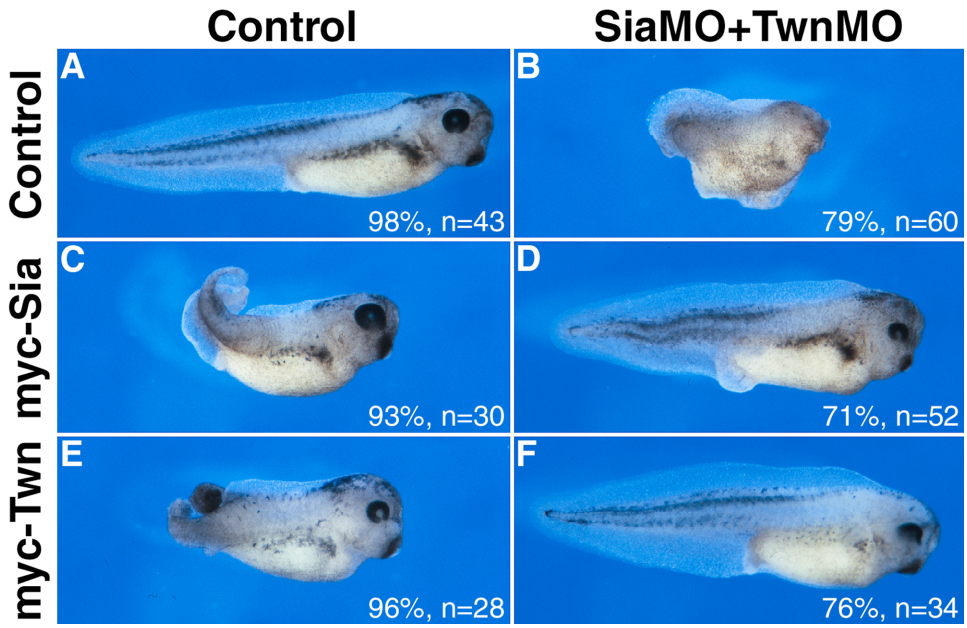


Figure 2.9 Rescue of axial development in the Siamois-Twin double knockdown embryo.

(B,D,F) At the 4-cell stage both dorsal blastomeres were injected with a combination of the Sia and Twn oligonucleotides (SiaMO+TwnMO, 25ng+25ng). At the 8-cell stage a single dorsal blastomere was injected with 50pg of (C,D) myc-Sia or (E,F) myc-Twn. myc-Sia and myc-Twn fully rescued axial development in double knockdown embryos (D,F), and resulted in mild dorsalization in control embryos (C,E). Whole embryo morphology (dorsal up, anterior right) is shown at the tailbud stage, with percentage of embryos displaying the representative phenotype and total embryos analyzed indicated in the lower right for each panel. (A) Uninjected control embryo.

2.8L'). These gene expression defects indicate a dramatic loss of organizer formation at the early gastrula stage, and are consistent with the severity of the axial defects observed later in development. In contrast, the direct Wnt target, Xnr3, is unaffected by Sia/Twn knockdown (data not shown). The results indicate that Sia and Twn together are essential regulators of organizer formation and subsequent axial development. Furthermore, we find that in response to loss-of-function for either Sia or Twn, the individual proteins can functionally compensate and support normal development.

2.3.7 Siamois and Twin are required to mediate Xwnt8-induced axis induction

Sia and Twn expression is activated in response to maternal Wnt signals at the midblastula transition, and multiple Tcf binding sites within the Sia and Twn promoters mediate direct activation by ßcatenin (Brannon et al., 1997; Brannon and Kimelman, 1996; Carnac et al., 1996; Fan et al., 1998; Laurent et al., 1997; Nelson and Gumbiner, 1998). Previous reports suggest that Sia is required downstream of both maternal Wnt signals and ßcatenin in axis induction (Fan and Sokol, 1997; Kessler, 1997), and Sia and Twn function are required for LiCl-mediated dorsalization of the embryo (Ishibashi et al., 2008). Furthermore, the axial defects we report for Sia-Twn knockdown are those predicted for inhibition of maternal Wnt signaling (reviewed in Heasman, 2006). The requirement for Sia and Twn in mediating the response to maternal Wnt signaling was determined by examining the influence of Sia-Twn knockdown on Xwnt8-induced axis induction (Fig. 2.10). At the four-cell stage, both ventral blastomeres were injected with Sia MO or Twn MO individually, or with the combination of both MOs, and at the eight-cell stage a single ventral blastomere was injected with Xwnt8 mRNA. Complete axis formation was induced at high frequency in response to Xwnt8 (90%) (Fig. 2.10B), and

Figure 2.10 Siamois and Twin are required for Xwnt8 induction of ectopic axis formation.

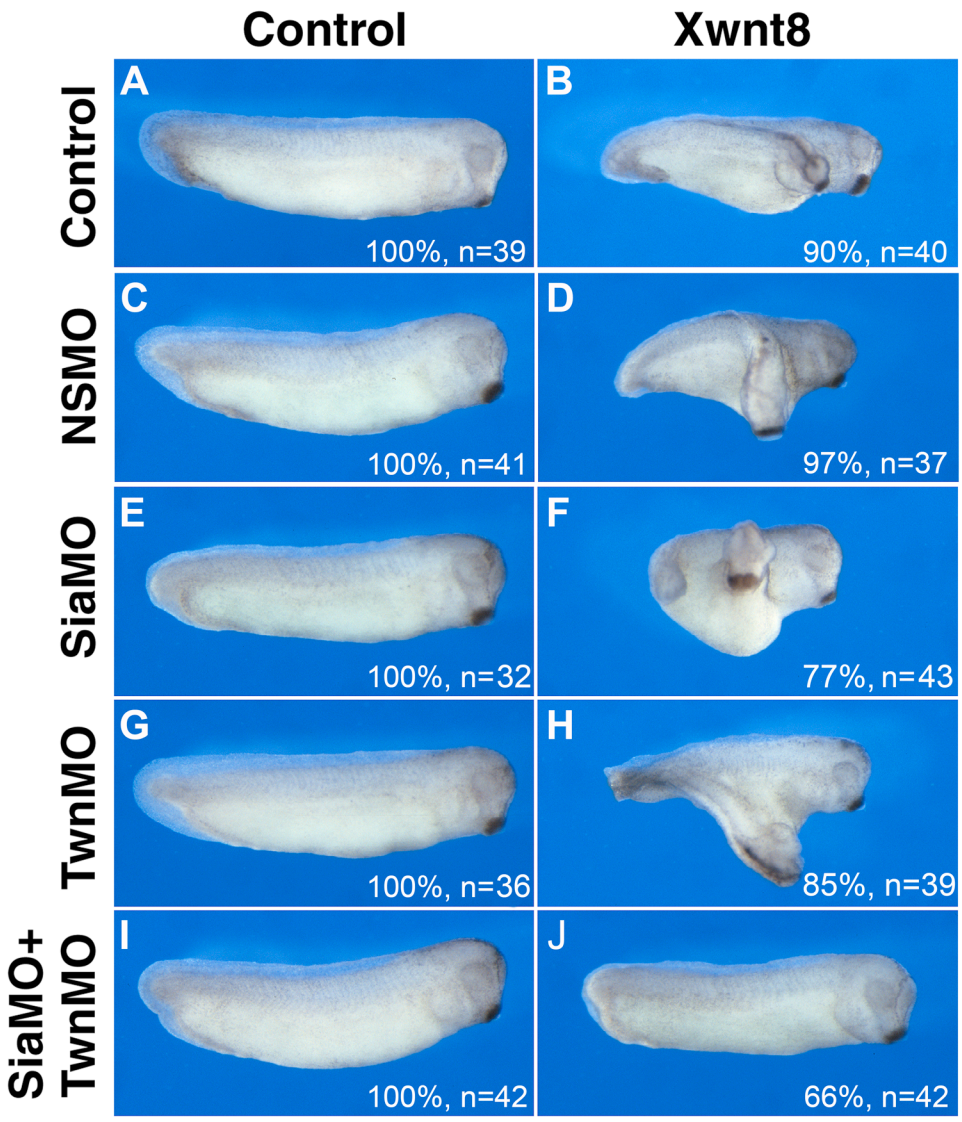


Figure 2.10 Siamois and Twin are required for Xwnt8 induction of ectopic axis formation.

At the 4-cell stage both ventral blastomeres were injected with (C,D) a non-specific control morpholino oligonucleotide (NSMO, 50ng), (E,F) a Sia-specific oligonucleotide (SiaMO, 25ng), (G,H) a Twn-specific oligonucleotide (TwnMO, 25ng), or (I,J) a combination of the Sia and Twn oligonucleotides (SiaMO+TwnMO, 25ng+25ng). (B,D,F,H,J) At the 8-cell stage a single ventral blastomere was injected with Xwnt8 mRNA (5pg). (A-J) Whole embryo morphology (dorsal up, anterior right) is shown at the tailbud stage, with percentage of embryos displaying the representative phenotype and total embryos analyzed indicated in the lower right for each panel. (A) Uninjected control embryo.

this response was unaffected by Sia MO, Twn MO or the non-specific control MO (77%, 85%, and 97%, respectively) (Fig. 2.10D,F,H). Simultaneous knockdown of Sia and Twn abrogated Xwnt8-mediated axis induction in most embryos (66% normal development) (Fig. 2.10J), with 34% displaying a weaker partial axis induction, while none of the embryos displayed a complete ectopic axis (data not shown). These data may reflect a partial inhibition of Xwnt8 activity, which could suggest an incomplete knockdown of Sia and Twn, as well as the presence of other effectors of the Wnt pathway in organizer formation. We note that ventral injection of the MOs alone had no effect on axial development (Fig. 2.10C,E,G,I). The results indicate that Sia and Twn together are required for Wnt induction of axis formation, demonstrating an essential and redundant role for Sia and Twn in mediating the transcriptional response to maternal Wnt signaling in axis formation.

2.4. Discussion

Our results demonstrate an essential role for Sia and Twn in the transcriptional activation of *Gsc* and the formation of the Spemann organizer in *Xenopus*. Sia and Twn form functionally equivalent homodimers and heterodimers that occupy a conserved Wnt-responsive element of the *Gsc* promoter. Knockdown of Sia and Twn together, but not individually, results in severe axial defects, characterized by a loss of organizer gene expression and a failure of organizer formation. The results demonstrate that Sia and Twn are functionally redundant, as predicted from their structural, expression profile and functional similarities, and together are essential for formation of the Spemann organizer. Furthermore, Sia and Twn are required transcriptional mediators of the response to maternal Wnt signals in organizer formation and axial development. These

studies establish Sia and Twn as essential and redundant activators of the Spemann organizer transcriptional program in the *Xenopus* gastrula.

2.4.1 Siamois and Twin are essential for Spemann organizer formation

Sia and Twn show striking similarity in structure, expression pattern, transcriptional activity and developmental function. With nearly identical homeodomains (88% identity) (Laurent et al., 1997; Lemaire et al., 1995), Sia and Twn likely bind to and activate a common set of target genes within the organizer domain of the early gastrula. Our results demonstrate that Sia and Twn transactivate target genes as homodimers or heterodimers with equivalent function. In gain-of-function studies, ventral expression of Sia or Twn induced a complete axial duplication containing head and trunk structures (Laurent et al., 1997; Lemaire et al., 1995). Taken together, these observations predict that Sia and Twn function redundantly in the regulation of organizer formation.

Previous developmental studies of Sia and Twn are consistent with redundant and essential roles in organizer formation, but did not provide a definitive analysis. Overexpression of a dominant repressive form of Sia (Eng-Sia), fully inhibits organizer gene expression, resulting in disruption of head, trunk and tail structures, consistent with complete ventralization of the body axis (Fan and Sokol, 1997; Kessler, 1997). At the time of these studies, Twn had not yet been identified, but it is predicted that Eng-Sia strongly represses the common targets of both Sia and Twn, and therefore, the phenotypic response to Eng-Sia likely reflects the consequence of interfering with both Sia and Twn function. Given the overexpression of a dominant repressive fusion protein, it is possible that the severity of the development defects reflects off-target effects. However, the complete rescue of axis formation by coexpression of native Sia argues for

specificity in the phenotypic defects obtained (Fan and Sokol, 1997; Kessler, 1997). While not true loss-of-function analyses, these studies support an essential role for Sia and Twn in organizer formation and axial development.

In contrast to the Eng-Sia studies, a recent knockdown analysis of Sia and Twn demonstrated redundancy, but a requirement only for anterior axial development (Ishibashi et al., 2008). The conclusion that Sia and Twn are required for head, but not trunk formation, suggested that Sia and Twn are not required for the full activity of the Spemann organizer. The knockdown results we obtained are consistent with the Eng-Sia studies (Fan and Sokol, 1997; Kessler, 1997), but not with the prior knockdown analyses (Ishibashi et al., 2008). We find that knockdown of both Sia and Twn results in a complete loss of organizer formation (Fig. 2.8H',I'), and consequently neither head nor trunk structures form in the most severe phenotypic class (Fig. 2.8J). The discrepancy in the severity of axial defects likely reflects a difference in knockdown efficiency, with the prior results representing a partial loss-of-function for Sia and Twn, while in our studies a more complete knockdown was achieved. In support of this interpretation, we find that lower dosage of the mixture of Sia MO and Twn MO phenocopies the anterior defects previously reported (Fig. 2.8M). Therefore, our results confirm that Sia and Twn are redundant and essential for formation of the Spemann organizer, including both head and trunk organizer activity.

Sia and Twn are redundant factors, and together are essential for the formation of the Spemann organizer. Sia and Twn appear to play equivalent roles in organizer formation, as knockdown of either Sia or Twn alone has no effect on axis formation (Fig. 2.8E,F). This suggests that Sia or Twn homodimers can compensate for the loss of the Sia-Twn heterodimer. The overall structure of Sia and Twn are highly similar, with high

sequence conservation with their homeodomains, as well as within small regions N-terminal to the homeodomain (Laurent et al., 1997). It is likely that Sia and Twn were formed as a result of the duplication of an ancestral Sia-Twn-like gene, whether a local or genome-wide duplication, but despite significant sequence divergence outside of the homeodomain, it appears that the transcriptional and developmental functions of these genes have not diverged (Van de Peer et al., 2009). Further studies may reveal whether Sia and Twn have discrete functions, perhaps in a target-specific or context-specific manner.

An intriguing observation is the apparent absence of Sia and Twn orthologs in non-amphibian vertebrates. While Sia and Twn orthologs have been identified in the closely related amphibian *Xenopus tropicalis*, other vertebrate orthologs have yet to be identified despite extensive efforts. Given the presence of the conserved Sia-Twn response element in all vertebrate *Gsc* promoters, the apparent absence of Sia and Twn orthologs raises questions about the conservation of Sia and Twn and the role of functional homologs in organizer formation of other vertebrates. Interestingly, a similar conundrum is found in zebrafish *bozozok*, a homeodomain protein that functions as a transcriptional repressor (Yamanaka et al., 1998; Fekany et al., 1999; Koos and Ho, 1999). *bozozok* is essential for organizer formation and expression of organizer genes such as *gsc* and the Nodal-related gene, *squint* (Shimizu et al., 2000; Solnica-Krezel and Driever, 2001), yet no vertebrate orthologs have been identified. While true orthologs of Sia, Twn or Bozozok may be identified in other vertebrates, it seems likely that the developmental functions of these *Xenopus*- and zebrafish-specific factors may reside in functional homologs that are employed in other species to regulate organizer formation and organizer gene expression. The presence of species-specific transcriptional

regulators of organizer formation in *Xenopus* and zebrafish suggests an unexpected regulatory diversity, perhaps reflecting either distinct developmental demands in these species or an evolutionary flexibility at this discrete step of organizer formation.

2.4.2 Transcriptional regulation of Goosecoid and other organizer genes by Siamois and Twin

Our results suggest that Sia and Twn regulate *Gsc* transcription by binding to a conserved HD binding site within the Wnt responsive proximal element of the *Gsc* promoter. As direct targets of maternal Wnt signals (Brannon and Kimelman, 1996; Carnac et al., 1996; Crease et al., 1998; Fan et al., 1998; Nelson and Gumbiner, 1998; Nishita et al., 2000), Sia and Twn are expressed at the onset of zygotic gene expression in the blastula (Blythe et al., 2010; Laurent et al., 1997; Lemaire et al., 1995), and likely play a role in the initiation of the expression of organizer genes at the onset of gastrulation. Consistent with this mechanism, *Gsc* and *Chd* expression is reduced or absent at the start of gastrulation in Sia/Twn knockdown embryos (Fig. 2.8H',I'). The BMP antagonists Chordin and Noggin, which are required for proper organizer function (Khokha et al., 2005), can partially rescue axis formation in Sia/Twn knockdown embryos (data not shown), placing Chordin and Noggin downstream of Sia and Twn in Spemann organizer function.

The *Gsc* promoter also contains a highly conserved Nodal-responsive element (DE) in addition to the Wnt-responsive element (PE) (Watabe et al., 1995). Our results provide strong evidence that Sia and Twn mediate the zygotic response to maternal Wnt signals through direct binding to a conserved P3 site within the PE element of the *Gsc* promoter. However, which Nodal effectors are involved in the initiation of *Gsc* expression

and how those may interact with the Wnt effectors Sia/Twn remains to be determined. The Nodal signaling pathway has been shown to signal through several pathway effectors, including Fast1 (FoxH1), a Fox family transcription factor that is maternally expressed throughout the embryo (Chen et al., 1996), and Mix family members such as Mixer or Milk, which are paired-type homeodomain transcriptional activators that are zygotically expressed throughout the endoderm (Germain et al., 2000). Fast1 is present prior to and during gastrula stages (Watanabe and Whitman, 1999), suggesting that it likely plays a role in initiation of *Gsc* expression, perhaps in cooperation with Sia and Twn. Consistent with this idea, maternal knockdown of Fast1 results in decreased expression of *Gsc* (Kofron et al., 2004a), and Fast1 has been shown to directly occupy the endogenous *Gsc* promoter (Blythe et al., 2009). Mixer and Milk interact with the signaling mediator Smad2 in a Nodal-dependent manner, and can form a complex on the DE of the *Gsc* promoter (Germain et al., 2000). The zygotic expression of Mix family members suggests a later role in the maintenance of *Gsc* expression.

The Nodal-responsive DE and the Wnt-responsive PE are nearly adjacent (~50 bp separation) in all *Gsc* promoters (Watabe et al., 1995), raising the possibility that transcriptional effectors of the two pathways may interact or cooperate to activate *Gsc* transcription. Our preliminary results indicate that Nodal and Wnt pathway effectors synergistically enhance transcription of *Gsc* (Reid and Kessler, unpublished results), consistent with an interaction of pathway effectors at the *Gsc* promoter. The strong conservation of both the DE and the PE in vertebrate *Gsc* promoters suggests a conserved mechanism of *Gsc* regulation involving transcriptional integration of Nodal and Wnt signaling inputs.

Given the conserved structure of the *Gsc* promoter, it is interesting to consider

whether the function of Gsc is conserved across species. Disruption of Gsc function in *Xenopus*, either by knockdown or expression of a dominant activating form of Gsc, leads to severe anterior defects, including a reduction or loss of head structures anterior to the hindbrain (Sander et al., 2007; Yao and Kessler, 2001). In contrast, a mouse knockout of *Gsc* results in no developmental defects associated with organizer function (Rivera-Perez et al., 1995; Wakamiya et al., 1998; Yamada et al., 1995; Zhu et al., 1998). *Gsc* mutant mice gastrulate normally and show normal development of the primary body axes. However, the mutants do die shortly after birth due to severe craniofacial defects, as well as improperly formed sternum and ribs (Rivera-Perez et al., 1995). If the function of Gsc is not conserved in higher vertebrates, it remains to be seen whether the regulatory control of *Gsc* expression is conserved. The P3 site within the *Gsc* promoter is conserved in vertebrates (Fig. 2.1A), indicating that a paired-type homeodomain-containing protein likely regulates the expression of *Gsc* in all vertebrates. However, the identity of such proteins, their role in the initiation and/or maintenance of *Gsc* transcription, and their ability to mediate the transcription response to Wnt signals remain unknown. The mouse PE is Wnt-responsive in *Xenopus* explants (Watabe et al., 1995), suggesting that Wnt pathway inputs may influence the control of *Gsc* expression in mammals, but whether the PE confers Wnt-responsiveness in mammals remains to be determined. The availability of complete genome sequences and the introduction of powerful computational approaches should aid in the identification of *Gsc* regulators that may serve as the functional homologs of Sia and Twn in higher vertebrates.

Sia and Twn have been identified as direct regulators of *Gsc*, and likely mediate the Wnt-dependent transcriptional activation of multiple organizer genes (Fan and Sokol, 1997; Kessler, 1997; Laurent et al., 1997; Yamamoto et al., 2003). Sia, in cooperation

with other paired-type homeodomain proteins, has been implicated in the transcription of several organizer genes, including *Cerberus* (Yamamoto et al., 2003) and *Crescent* (Shibata et al., 2000). However, it is unclear how Sia may be interacting with other homeodomain proteins to affect gene transcription for other organizer-specific genes. Xlim-1 and Lim Domain Binding Protein-1 were shown to influence *Gsc* transcription, although through a site upstream of the PE (Mochizuki et al., 2000). Whether Sia and Twn initiate expression of *Gsc* and other organizer genes in cooperation with Nodal signals (Engleka and Kessler, 2001) remains to be determined, as is the role of other promoter elements and regulatory proteins that maintain organizer gene expression through the gastrula and neurula stages.

Formation of the organizer domain within the gastrula embryo is essential for germ layer patterning and axial development. Sia and Twn act redundantly downstream of the Wnt pathway to regulate formation of the organizer. Sia and Twn, and likely other factors, play an essential role in specifying the proper spatial and temporal expression of the organizer-specific gene *Gsc*. As mediators of the transcriptional response to maternal Wnt signals, and through cooperative interactions with other pathways, Sia and Twn control the expression of multiple organizer genes, thus contributing to the establishment of the organizer transcriptional program.

Chapter 3 Transcriptional Integration of Wnt and Nodal Signals in the Establishment of the Spemann Organizer

Christine Reid, Yan Zhang, Michael D. Sheets and Daniel S. Kessler*

3.1. Summary

Signaling inputs from multiple pathways are essential for the establishment of distinct cell and tissue types in the embryo. Therefore, multiple signals must be integrated to activate gene expression and confer cell fate, but little is known about how this occurs at the level of target gene promoters. During early embryogenesis, Wnt and Nodal signals are required for formation of the Spemann organizer, which patterns the primary germ layers and the body axis. Here, we demonstrate the transcriptional cooperation between the Wnt and Nodal pathways in the activation of three organizer genes, *Goosecoid*, *Cerberus* and *Chordin*. At the blastula stage, the Wnt pathway effectors Siamois and Twin and Nodal pathway effectors FoxH1 and Smad2/3 are co-expressed in a dorsal domain, preceding the expression of organizer genes in the gastrula. Wnt and Nodal pathway effectors synergize to strongly activate the transcription of these organizer genes. Effectors of both pathways occupy the *Goosecoid*, *Cerberus* and *Chordin* promoters and effector occupancy is enhanced with active signaling from both Wnt and Nodal. This suggests that, at organizer gene promoters, a stable transcriptional complex containing effectors of both pathways forms in response to Wnt and Nodal signaling. Consistent with this idea, the histone acetyltransferase, p300, is recruited to organizer promoters in a Wnt and Nodal effector-

* The data in section 3.3.2 and Figure 3.3 on mapping the regulatory domains of *Chordin*, was contributed by our collaborators, Yan Zhang and Michael D. Sheets at University of Wisconsin, Madison.

dependent manner. Taken together, these results offer a mechanism for spatial and temporal restriction in organizer gene transcription by the integration of two distinct signaling pathways, thus establishing the Spemann organizer domain.

3.2. Introduction

Wnt and Nodal signals are required for formation of the Spemann organizer, which is essential for germ layer patterning and axis formation (reviewed in De Robertis et al., 2000). Wnt and Nodal pathways are required for the expression of several organizer genes, including *Goosecoid* (*Gsc*), *Cerberus* (*Cer*), and *Chordin* (*Chd*) (Agius et al., 2000; Crease et al., 1998; Engleka and Kessler, 2001; Heasman et al., 1994; Hoodless et al., 1999; Miller et al., 1999; Osada and Wright, 1999; Watanabe and Whitman, 1999; Wylie et al., 1996; Yang et al., 2002), suggesting that integrated signaling from these pathways promotes organizer gene expression. The presence of a Wnt responsive Proximal Element (PE) and a Nodal responsive Distal Element (DE) within the *Gsc* promoter suggests that Wnt and Nodal signals may be integrated at the level of transcription (Watabe et al., 1995). The close proximity of the PE and the DE suggests that Wnt and Nodal effectors could interact to activate *Gsc* expression (Watabe et al., 1995). Consistent with this idea, the *Cer* promoter contains several homeodomain binding sites that mediate a cooperative response to Wnt and Nodal (Yamamoto et al., 2003). Therefore, the transcription of multiple organizer genes is dependent on the integration of Wnt and Nodal signals, yet how these signals are integrated is unknown.

Maternal Wnt signals activate expression of two homeodomain proteins, Siamois (Sia) and Twin (Twn), transcriptional activators that are essential for organizer gene expression and axis formation (Bae et al., 2011; Brannon et al., 1997; Brannon and

Kimelman, 1996; Carnac et al., 1996; Crease et al., 1998; Fan et al., 1998; Fan and Sokol, 1997; Ishibashi et al., 2008; Kessler, 1997; Kodjabachian and Lemaire, 2001; Kodjabachian and Lemaire, 2004; Laurent et al., 1997; Lemaire et al., 1995) Overexpression of Sia or Twn within ventral mesoderm induces expression of *Gsc*, *Cer*, *Chd* (Kessler, 1997; Kodjabachian and Lemaire, 2001), and Sia and Twn regulate transcription of *Gsc* (Bae et al., 2011; Laurent et al., 1997). Nodal signals through maternal FoxH1 and Smad2/3 to activate expression of mesodermal and organizer genes, including *Gsc*, *Cer*, and *Chd* (Saka et al., 2007; Watanabe and Whitman, 1999). Knockdown of maternal FoxH1 results in a loss of organizer gene expression (Kofron et al., 2004a), while expression of a dominant negative Smad2 reduces expression of *Gsc*, *Chd* and *Cer* (Hoodless et al., 1999). FoxH1 directly binds the *Gsc* promoter (Blythe et al., 2009), suggesting that *Gsc* is a direct target of Nodal signaling. Taken together, these findings suggest that Wnt and Nodal effectors play an essential and direct role in the expression of several organizer genes.

Here, we demonstrate that Wnt effectors Sia/Twn and Nodal effectors FoxH1 and Smad2/3 cooperate to synergistically activate expression of *Gsc*, *Cer* and *Chd.* Sia/Twn and FoxH1 and Smad2/3 occupy the *Gsc*, *Cer* and *Chd* promoters. Active signaling from both pathways enhances occupancy of these effectors at organizer promoters, suggesting that a transcriptional complex forms at promoters when Wnt and Nodal are active. Sia/Twn or Nodal enhances occupancy of the histone acetyltransferase p300 at organizer promoters, suggesting that recruitment of co-factors contributes to organizer gene expression. Taken together, Wnt and Nodal pathway effectors form a transcriptional complex that synergistically activates expression of multiple organizer genes, providing a common mechanism for the robust transcription of organizer genes in

the gastrula.

3.3. Results

3.3.1. Wnt and Nodal synergistically activate organizer gene expression

The *Gsc* promoter contains a Wnt responsive PE and a Nodal responsive DE (Bae et al., 2011; Watabe et al., 1995) suggesting Wnt and Nodal may cooperate in the expression of *Gsc*. To assess the interaction of Nodal and Sia/Twn in *Gsc* regulation, we performed luciferase assays in *Xenopus* animal explants using the *Gsc* reporter (Watabe et al., 1995). Expression of Sia, Twn or Xnr1 in animal explants activated the Gsc reporter (6.4-fold, 5.3-fold and 4.7-fold, respectively) (Fig. 3.1A) (Fan and Sokol, 1997; Kessler, 1997; Laurent et al., 1997; Watabe et al., 1995). Co-expression of Sia and Xnr1 or Twn and Xnr1 resulted in a synergistic activation of transcription (48.8-fold for Sia and Xnr1, 36.3-fold for Twn and Xnr1) (Fig. 3.1A). The synergy observed suggests that Sia/Twn and Nodal pathway effectors are interacting to enhance *Gsc* expression.

To determine whether Wnt and Nodal synergistically activate organizer gene expression, we performed quantitative reverse transcriptase PCR (QRT-PCR) for *Gsc, Cer* and *Chd* in animal explants. Expression of Sia, Twn or Xnr1 alone resulted in induced activation of *Gsc, Cer* and *Chd* (7-21.7 fold for Sia, 3-18.5 fold for Twn and 30-638 fold for Xnr1) (Fig. 3.1B-G). Co-expression of Sia and Xnr1 or Twn and Xnr1 resulted in a synergistic increase of *Gsc*, *Cer* and *Chd* expression(1333-2501-fold for *Gsc*, 445-865-fold for *Cer* and 90-115-fold for *Chd*) (Fig 3.1B-G). These data demonstrate a cooperative interaction between Sia/Twn and Nodal in activating transcription of organizer genes.

Figure 3.1 Nodal and Wnt synergistically activate organizer gene transcription.

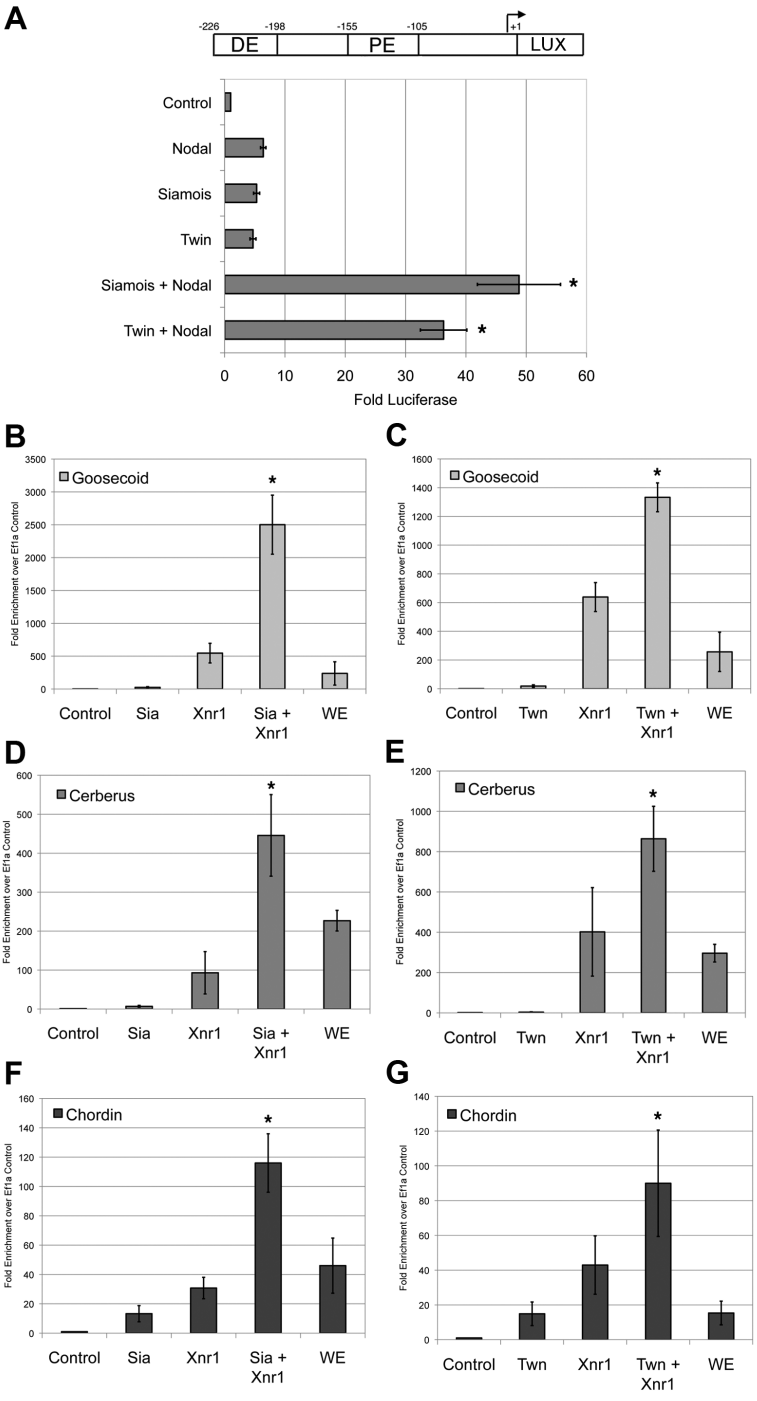


Figure 3.1: Nodal and Wnt synergistically activate organizer gene transcription. (A) One-cell stage embryos were injected with 50pg of Sia, Twn or Xnr1 (Nodal) mRNAs, or a mixture of Sia (50pg) + Xnr1 (50pg) or Twn (50pg) + Xnr1 (50pg). At the two-cell stage plasmid encoding *Gsc* reporter (100pg; diagrammed in A) was injected with CMV-Renilla Luciferase (10pg). The mean increase in luciferase activity and standard error for nine independent experiments is presented. (B-G) Analysis of *Gsc* (B,C), *Cer* (D,E) or *Chd* (F,G) transcript expression in animal cap explants in response to injection of (B,D,F) 50pg Sia, 50pg Xnr1, or Sia (50pg) + Xnr1 (50pg) or (C,E,G) 50pg Twn, 50pg Xnr1, or Twn (50pg) + Xnr1 (50pg). Animal explants were analyzed by quantitative RT-PCR at the gastrula stage for the expression of *Gsc*, *Chd* or *Cer* normalized to *Ef1*α. Control represents uninjected animal explants and WE represents intact embryos * indicates p value <0.05 as compared to the Sia, Twn and Xnr1 conditions. Data represent six independent experiments. Identical reactions without reverse transcriptase served as negative control (data not shown).

Expression patterns of Wnt and Nodal effectors support a role for these effectors in endogenous organizer gene expression (Blumberg et al., 1991; Bouwmeester et al., 1996; Chen et al., 1996; Germain et al., 2000; Saka et al., 2007; Sasai et al., 1994; Schohl and Fagotto, 2002). To confirm this, we examined effector expression by whole mount in situ hybridization or immunohistochemistry. Smad2/3 is ubiquitously expressed (Fig. 3.2A-C), with a bias of nuclear and cytoplasmic protein distribution to the dorsal side of the gastrulating embryo (Fig. 3.2B-C). Transcripts of FoxH1 are ubiquitously distributed at the blastula and gastrula stages (Fig. 3.2D-F) . The Wnt effectors Sia and Twn are expressed in the dorsal marginal zone prior to gastrulation (Fig. 3.2G, H) and at the dorsal blastopore lip at the early gastrula stage (Fig. 3.2I, J). *Gsc*, *Cer* and *Chd* are expressed at the dorsal blastopore lip and expression extend to the blastocoel floor in the deep marginal zone (Fig. 3.2K-P) . Therefore, Wnt and Nodal effectors are expressed in a region of overlap that corresponds to the subsequent location of organizer gene expression.

3.3.2. Identification of the Chd Regulatory Domain

The organizer gene *Chd* has an expression profile similar to *Gsc* and *Cer* and is dependent on both Wnt and Nodal signal for proper expression (Bae et al., 2011; Hoodless et al., 1999; Kofron et al., 2004a). We sought to identify the regulatory region of *Chd* to determine if it integrates Wnt and Nodal signals in a similar way to *Gsc* and *Cer*. A 1.2kB region upstream of the *Chd* transcriptional start site recapitulated organizer specific expression as visualized by GFP (data not shown). A plasmid containing a luciferase reporter downstream of the *Chd* -1.2kb was expressed in both dorsal (organizer) and ventral (non-organizer) blastomeres and the fold activation was

Figure 3.2 Expression patterns of Wnt and Nodal effectors and organizer genes in embryos bisected along the dorsal/ventral axis.

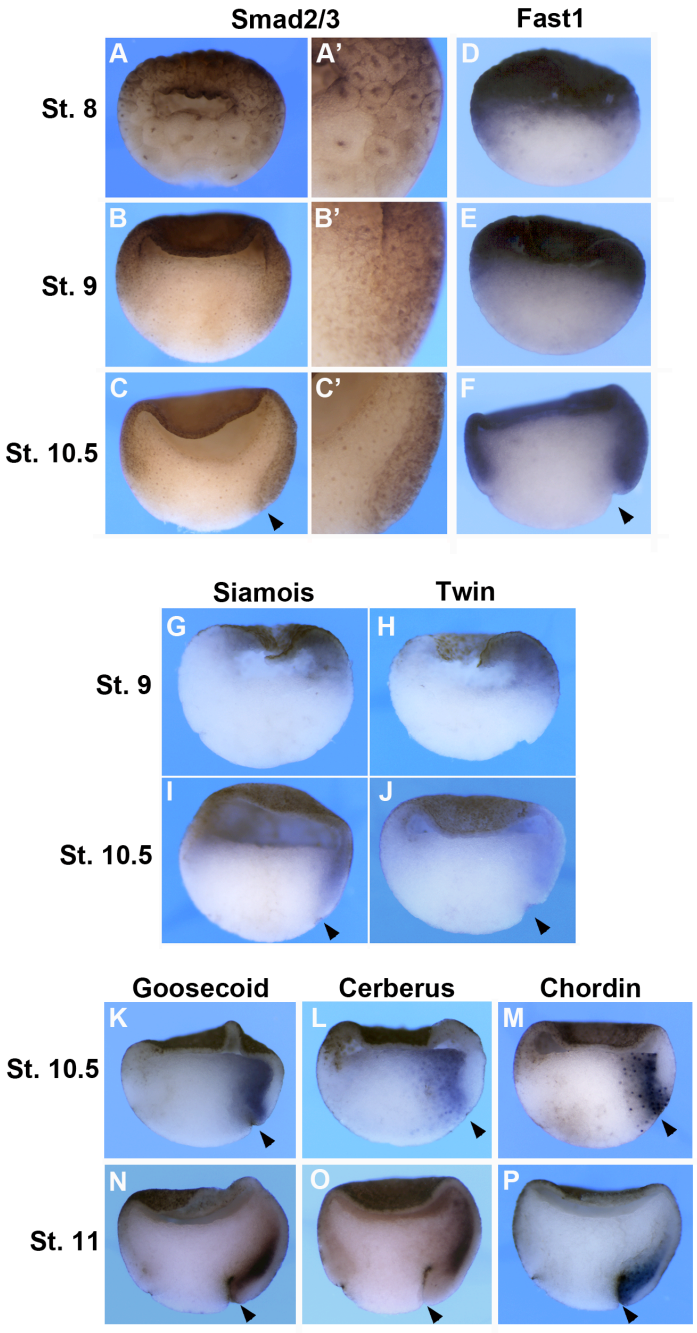


Figure 3.2: Expression patterns of Wnt and Nodal effectors and organizer genes in embryos bisected along the dorsal/ventral axis.

(A-C') Antibody staining for total Smad2/3 protein in stage 8 (A-A'), stage 9 (B-B') and stage 10.5 (C-C') embryos. Panels A', B', and C' show an enhanced view of the embryos in E, F, G, respectively. (D-F) Expression of FoxH1, as seen by in situ hybridization, during stage 8 (D), stage 9 (E) and stage 10.5 (F) We note that FoxH1 transcript was detected throughout the embryo, including in the vegetal region. (G-J) Expression of Sia or Twn transcript, as seen by in situ hybridization, during stage 9 (G-H)and stage 10.5 (I-J) of embryogenesis. (K,N) Expression of Gsc transcript as seen by in situ hybridization, on stage 10.5 (K) and Stage 11 (N) embryos. (L,O) Expression of Cer transcript as seen by in situ hybridization, in Stage 10.5 (L) and Stage 11 (O) embryos. (M,P) Expression of Chd transcript as seen by in situ hybridization in Stage 10.5 (M) and Stage 11 (P) embryos. The black arrowhead indicates the dorsal blastopore lip.

calculated (Fig. 3.3A). The -1.2kB had approximately a 14 fold increase in activity in the organizer domain, as compared to the non-organizer domain (Fig. 3.3A). Deletions from the 5' end of the promoter region were carried out to identify regulatory domains involved in organizer specific expression. Reporter activity was maintained in deletions up to -211, suggesting that areas between the start of transcription and -211bp regulate *Chd* expression in the organizer (Fig. 3.3A). A closer look at the *Chd* promoter between -211 and the start site of transcription revealed a P3 site beginning at -107 (Fig. 3.3C), which suggests that *Chd* expression in the organizer may be dependent on homeodomain proteins such as Sia/Twn, similar to both Gsc and Cer. Mutation of this P3 site (from TAAGTGCATTA to TCGGTGCACGA) abrogated organizer specific expression (Fig. 3.3A), indicating that Sia/Twn dependent expression of *Chd* may be mediated through this P3 site.

To determine whether the identified *Chd* expression is mediated by Sia, the *Chd* luciferase reporters were tested for responsiveness to Sia in ectodermal explants. Both the -1.2kB and the -211 *Chd* promoters responded to Sia overexpression (11-fold for -1.2kB*Chd* and 7-fold for -211*Chd*) (Fig. 3.3B). However, mutation of the P3 element prevented Sia activation (Fig. 3.3B), suggesting that the P3 element is required for Sia-mediated expression of *Chd*. Taken together, these results identify a regulatory domain which likely controls *Chd* expression in the organizer, and further show that *Chd* region. We also identified several putative FoxH1 binding sites in close proximity with the P3 site (Fig. 3.3C), suggesting that *Chd* may be regulated by both Wnt and Nodal signals, as has been found for both *Gsc* and *Cer*.

Figure 3.3 Mapping the Regulatory Domain of *Chordin*.

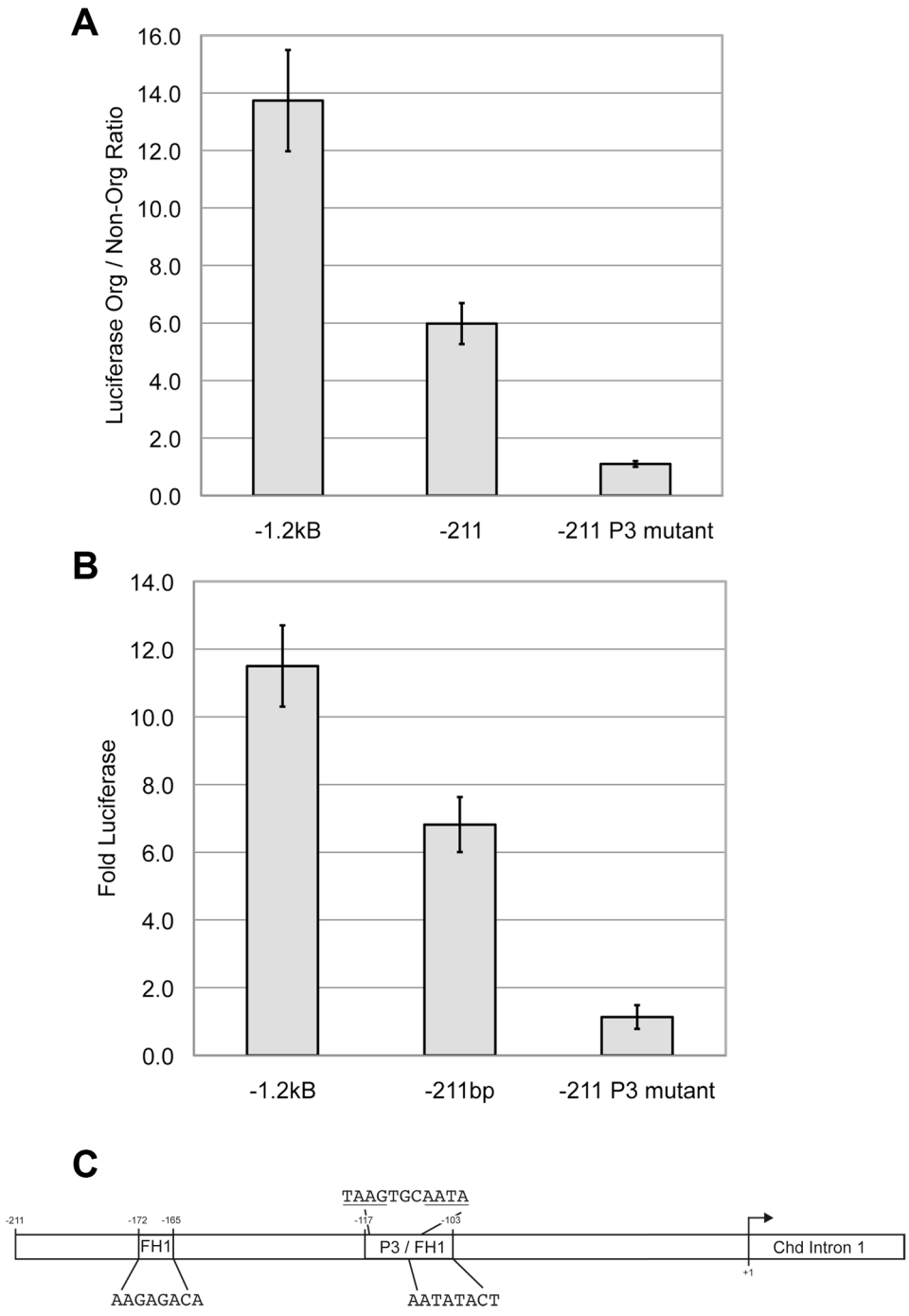


Figure 3.3: Mapping the Regulatory Domain of *Chordin*.

(A) In order to identify the regulatory domain of *Chd*, four-cell embryos were injected either in dorsal or ventral blastomeres with -1.2*Chd*, -211*Chd* or -211*Chd* P3 mutant luciferase reporters. To identify regions important in organizer expression of *Chd*, the fold luciferase was calculated, comparing dorsal expression to ventral expression. A loss of expression was observed when the P3 site was mutated. (B) One cell stage embryos we injected with 50pg of Sia. At the two-cell stage plasmid encoding -1.2*Chd*, -211*Chd* or -211*Chd* P3 mutant luciferase reporters were injected together with CMV-Renilla Luciferase. Animal explants prepared at the blastula stage were assayed for luciferase activity at the midgastrula stage. Values shown are normalized to Renilla luciferase activity. The mean increase in luciferase activity and standard error for three independent experiments is presented here. (C) Diagram of the -211 *Chd* promoter region, with putative FoxH1 (FH1) sites and P3 site highlighted. The FoxH1 site sequences are highlighted below the diagram, while the P3 sequence is highlighted above the diagram, with the homeodomain half sites underlined.

3.3.3. Wnt and Nodal Effectors Occupy Organizer Promoters

Cooperation between Wnt and Nodal pathways in organizer gene activation suggests that pathway effectors directly bind these promoters to activate transcription. For *Gsc*, the close proximity of Wnt and Nodal response elements implies that Wnt and Nodal effectors occupy the *Gsc* promoter, allowing functional interactions. To determine whether Sia, Twn and Nodal pathway effectors regulate *Gsc*, whole embryo chromatin immunoprecipitation (ChIP) was performed in the early gastrula. Myc-Sia or myc-Twn expressing embryos were collected and fixed at early gastrula stage (stage 10.25). Immunoprecipitation was performed for the myc-tag and quantitative PCR (QPCR) assessed recovery of the *Gsc* promoter. Sia and Twn occupy the *Gsc* promoter (Fig. 3.4A) (Bae et al., 2011) and do not occupy genomic *Xmlc2*, demonstrating direct regulation of *Gsc* by Sia and Twn (Fig. 3.4A). Sia specifically binds the *Gsc* promoter, as a DNA-binding inactive form of Sia (SiaQ191E) did not occupy the *Gsc* promoter (Fig. 3.4A). Myc-FoxH1 occupied the *Gsc* promoter, both in the absence and presence of Xnr1 (Fig. 3.4B). ChIP with an antibody detecting endogenous Smad2/3 revealed occupancy at the *Gsc* promoter that is significantly increased in response to Xnr1 (Fig. 3.4C). The results demonstrate that both Wnt and Nodal effectors are present at the *Gsc* promoter, consistent with direct regulation of *Gsc* by Wnt and Nodal.

For *Cer*, functional Sia/Twn response elements have been identified within proximal promoter sequence (Yamamoto et al., 2003) The expression pattern of *Chd* is similar to *Gsc* and *Cer*; we therefore sought to identify Sia/Twn response element in the *Chd* promoter. Indeed, the *Chd* promoter contains a Sia-responsive element within the proximal promoter (Figure 3.3). Consistent with direct regulation of the expression of *Cer* and *Chd*, Sia and Twn occupy *Cer* and *Chd* promoters (Fig. 3.4D,G), but SiaQ191E did

Figure 3.4 Nodal and Wnt effectors occupy organizer promoters

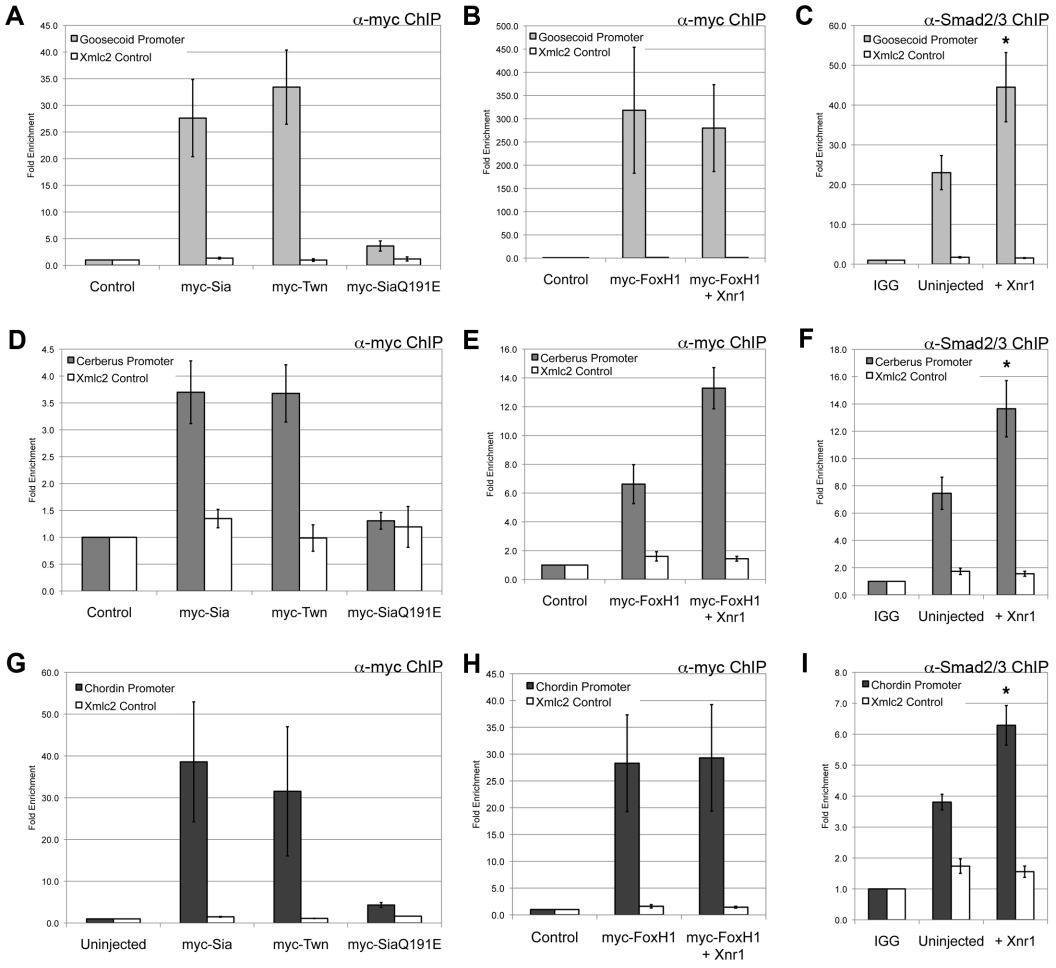


Figure 3.4: Nodal and Wnt effectors occupy organizer promoters.

(A,D,G) Genomic regions recovered by chromatin immunoprecipitation (ChIP) for myc-Sia, myc-Twn or DNA binding dead Sia (myc-SiaQ191E) were evaluated by quantitative PCR (QPCR) for (A) *Gsc*, (B) *Cer*, or (C) *Chd* promoters. Immunoprecipitation using anti-myc antibody was performed on uninjected embryos (Control). Data represent five independent experiments. (B,E,H) Genomic regions recovered by ChIP for myc-FoxH1, or myc-FoxH1 with 50pg Xnr1 mRNA (myc FoxH1+Xnr1) were evaluated by QPCR for (B) *Gsc*, (E) *Cer*, or (H) *Chd* promoters. Data represent three independent experiments. (C,F,I) Genomic regions recovered by ChIP for endogenous Smad2/3 in uninjected embryos or embryos expressing 50pg Xnr1 mRNA (+Xnr1) were evaluated by QPCR for (C) *Gsc*, (F) *Cer*, or (I) *Chd* promoters. Rabbit IGG added to uninjected embryo extract serves as a control (IGG). The white bars represent QPCR for genomic *Xmlc2* as a control. Data represent three independent experiments.* indicates p value <0.05 as compared to uninjected embryos.

not (Fig. 3.4D.G), demonstrating specific binding of Sia.

Expression of *Cer* and *Chd* are dependent on Nodal signaling (Agius et al., 2000; Engleka and Kessler, 2001), and the *Cer* promoter contains Nodal and Wnt response elements (Yamamoto et al., 2003). The *Chd* promoter contains several putative FoxH1 binding sites (Fig. 3.3C), but a defined Nodal response element has not been identified. ChIP analyses were performed to determine whether Nodal effectors occupy *Cer* and *Chd* promoters. Myc-FoxH1 occupied the *Cer* and *Chd* promoters, both in the absence and presence of Xnr1 (Fig. 3.4E,H), and endogenous Smad2/3 occupied these promoters at elevated levels in response to Xnr1 (Fig. 3.4F,H). Taken together, these results confirm that Nodal regulates *Cer* and *Chd* expression through an element within the same region as the Sia/Twn response element, which implies that *Cer* and *Chd* regulation may be similar to *Gsc*. The close proximity of the Nodal and Wnt response elements in each promoter (Fig. 3.3C) (Watabe et al., 1995; Yamamoto et al., 2003), and the location of the response elements within 250bp of the start of transcription (this study; Watabe et al., 1995; Yamamoto et al., 2003) strongly argues for functional conservation in mediating the response to Wnt and Nodal. Furthermore, the presence of these effectors in close proximity at these promoters suggests potential functional interactions mediating the synergistic response to Wnt and Nodal.

3.3.4. Wnt and Nodal effectors Interact at Organizer Promoters

The synergistic response of organizer genes to Nodal and Wnt signals, and the proximity of Nodal and Wnt effectors occupying organizer gene promoters, suggest that physical and functional interactions between these effectors may occur to activate organizer gene transcription. The synergistic transcriptional response to Nodal and Wnt

signals may reflect formation of a transcriptional complex, containing both Wnt and Nodal effectors, which enhances effector occupancy and/or activity. To assess the possible interactions of Nodal and Wnt effectors we examined occupancy by Sia or Twn in the absence of exogenous Xnr1. For both Sia and Twn, occupancy of the *Gsc*, *Cer* and *Chd* promoters is significantly enhanced (2-4 fold) with addition of exogenous Xnr1 (Fig. 3.5A,C,E). We note that the influence of Nodal signaling on Sia and Twn occupancy was examined at increasing doses of Sia and Twn (1-50pg). At lower expression levels of Sia or Twn (1-25pg) enhanced occupancy is observed in response to Xnr1 (Fig. 3.5 and data not shown), while at higher expression levels of Sia and Twn (50pg; Figs. 3.1 and 3.4) the already strong occupancy was not enhanced.

To determine whether Sia/Twn influence occupancy of Nodal effectors, Smad2/3 and FoxH1, occupancy of the *Gsc*, *Cer*, and *Chd* promoters was examined in response to Sia or Twn expression in the presence or absence of Xnr1. Smad2/3 occupies the organizer promoters in control embryos (Figs. 3.5B,D,F), and occupancy is not increased in response to Sia or Twn alone. However, a significant increase in Smad2/3 occupancy is observed with Sia or Twn co-expression with exogenous Xnr1 (Fig. 3.5B,D,F). We observe no increase in FoxH1 occupancy at the organizer promoters in response to Sia or Twn (data not shown). Taken together, the results indicate that active phospho-Smad2/3 interacts, either directly or indirectly, with Sia and Twn at the organizer promoters, and this interaction results in a reproducible enhancement of occupancy for Sia, Twn and Smad2/3. This enhanced occupancy likely reflects the formation of a stable transcriptional complex, containing both Wnt and Nodal effectors, as well as other co-regulatory proteins. Assembly of such a stable transcription complex at organizer promoters may account for the synergistic activation of transcription in

Figure 3.5 Wnt and Nodal effectors form a transcriptional complex at organizer promoters

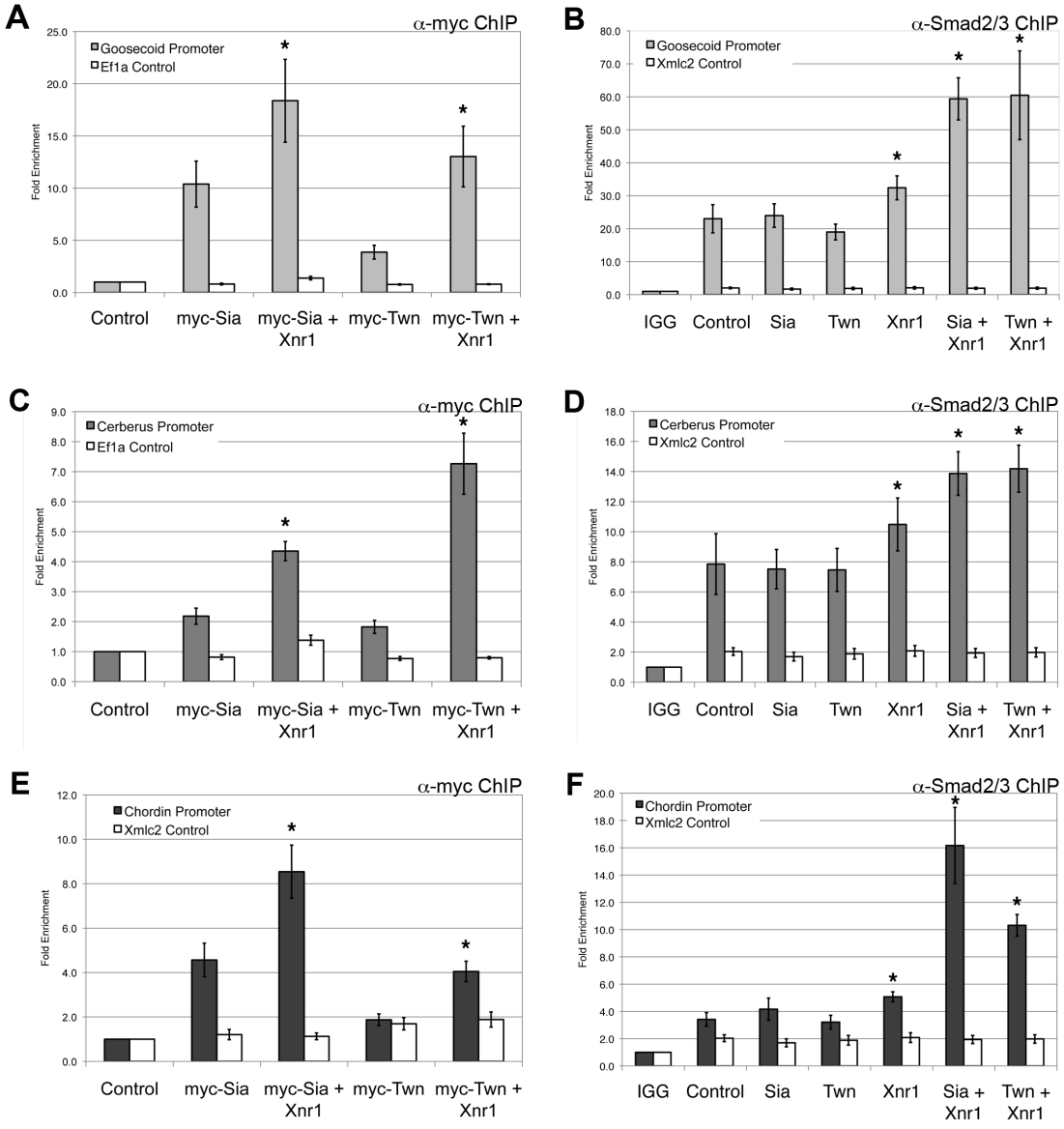


Figure 3.5: Wnt and Nodal effectors form a transcriptional complex at organizer promoters.

(A,C,E) Genomic regions recovered by ChIP for 10pg myc-Sia, 10pg myc-Sia + 50pg Xnr1, 10pg myc-Twn, or 10pg myc-Twn + 50pg Xnr1 were evaluated by QPCR for (A) the *Gsc*, (C) *Cer* or (E) *Chd* promoters. The white bars represent QPCR for genomic *EF1*α as a control. Data represent eight independent experiments. (B,D,F) Genomic regions recovered from ChIP for endogenous Smad2/3 in uninjected embryos (Control), or embryos expressing 50pg of Sia, Twn or Xnr1 or combinations of 50pg Sia + 50pg Xnr1 or 50pg Twn + 50pg Xnr1 were evaluated by QPCR for (B) *Gsc*, (D) *Cer*, or (F) *Chd* promoters. The white bars represent QPCR for genomic *Xmlc2* as a control. Smad2/3 association with the promoters is significantly enhanced (p value <0.05) in the presence of Xnr1 as compared to uninjected embryos, (* on the Xnr1 condition). Smad2/3 association with the promoters is further enhanced (p value <0.05) in the presence of Sia+Xnr1 or Twn+Xnr1 (indicated by *) as compared to individual Sia, Twn, or Xnr1. Data represent six independent experiments

response to Wnt and Nodal signals.

3.3.5. Wnt and Nodal effectors recruit p300 to target organizer promoters

The transcription complex that forms at organizer gene promoters may include common co-activators recruited by both Wnt and Nodal. The histone acetyltransferase, p300, is essential for *Gsc* and *Chd* expression (Kato et al., 1999) and results in enhanced transcription in response to Nodal (Inoue et al., 2007; Ross et al., 2006; Tu and Luo, 2007). To verify a functional interaction between Sia/Twn, Nodal and p300, we examined the requirement for p300 in Sia/Twn or Nodal-mediated activation of the *Gsc* reporter. While Nodal, Sia or Twn strongly activated the *Gsc* promoter (7-17 fold activation), E1A co-expression greatly inhibited that response (~2-fold activation), while E1AΔ2-36, which does not interact with p300, had no effect (Fig. 3.6A,B,C)(Frisch and Mymryk, 2002). Taken together, the results demonstrate that p300 is a required co-regulator in the activation of *Gsc* by Nodal and Sia/Twn.

The requirement for p300 in *Gsc* activation suggests that p300 is recruited to organizer promoters by Wnt and Nodal effectors. To examine p300 occupancy at organizer promoters, a myc-tagged form of *Xenopus* p300 was expressed alone or withSia, Twn or Xnr1. While p300 alone had low occupancy at the *Gsc*, *Cer* and *Chd* promoters, occupancy was significantly increased (2-4 fold) in the presence of Sia, Twn or Xnr1 (Fig. 3.6D-F). Therefore, Wnt and Nodal pathway effectors mediate recruitment of p300 to organizer gene promoters.

Figure 3.6 Wnt and Nodal effectors recruit p300 to organizer gene promoters

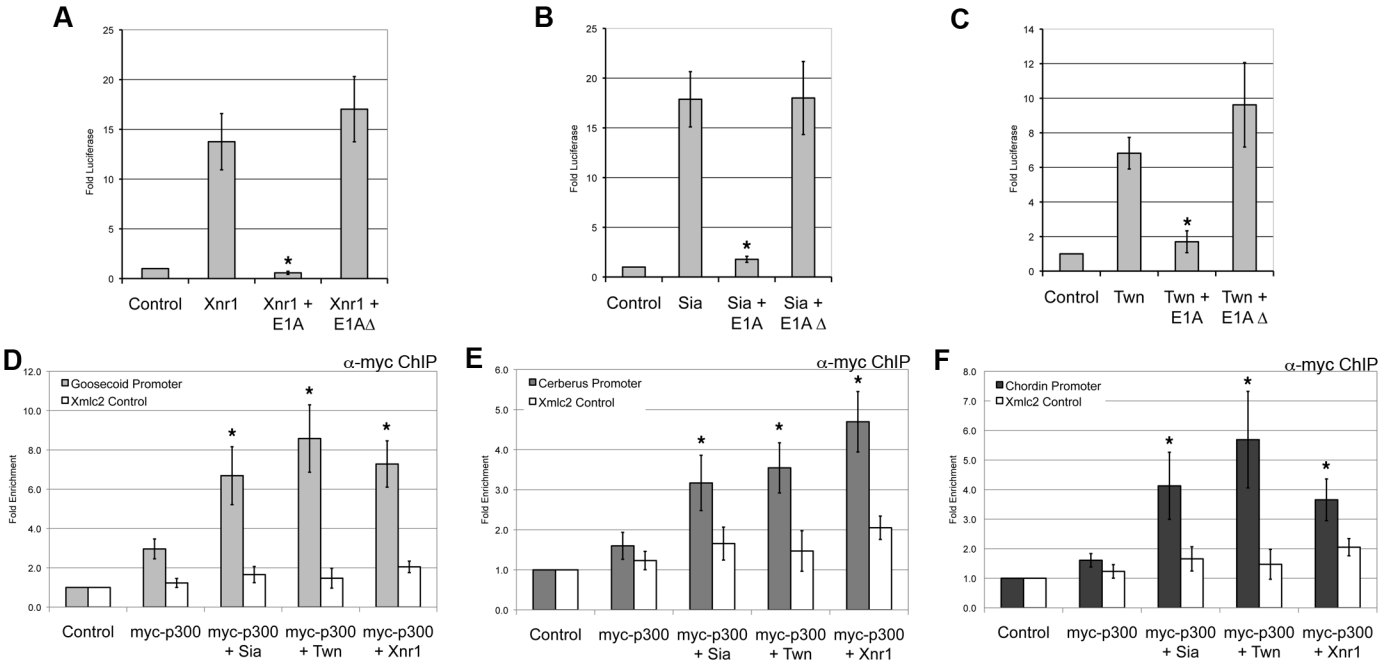


Figure 3.6: Wnt and Nodal Effectors Recruit p300 to Organizer Gene Promoters.

(A-C) At the one-cell stage the animal pole was injected with 50pg of Xnr1 (A), Sia (B) or Twn (C), either alone or with full length E1A or E1AΔ2-36 as a control. Two-cell embryos were injected with plasmid encoding *Gsc* reporter (100pg) with CMV-Renilla Luciferase (10pg). Data represent three independent experiments. The * indicates p value <0.05 as compared to Xnr1, Sia or Twn activation of *Gsc* reporter. (D-F) Genomic regions recovered by ChIP for myc-p300 either alone or with 150pg Sia or Twn or 50pg Xnr1 were evaluated by QPCR for (D) *Gsc*, (E)*Cer*, or (F) *Chd* promoters. The white bars represent QPCR for genomic *Xmlc2* as a control. * represents p<0.05 when compared to myc-p300 condition alone. Data represent six independent experiments

3.4. Discussion

The formation of the Spemann organizer depends on both Wnt and Nodal signals, which are active in the presumptive organizer domain at the blastula stage, prior to the onset of organizer gene transcription (reviewed in De Robertis et al., 2000). Cells within this domain receive both Wnt and Nodal signals, and integrate these inputs to generate temporally and spatially specific transcriptional responses. In the work presented here, we demonstrate that the Wnt and Nodal signaling inputs are directly received at multiple organizer gene promoters, and the physical and functional interaction among the pathway effectors results in strong transcriptional activation of the organizer genes. Transcriptional integration is accomplished by the assembly of a stable activating complex, containing Sia, Twn, FoxH1, Smad2/3, p300 and other components, at the promoters of *Gsc*, *Cer*, *Chd*, and likely additional organizer genes. We propose that in the late blastula, cells receiving both Wnt and Nodal inputs integrate these signals at the level of organizer gene promoters, establishing a temporally and spatially distinct transcriptional domain that results in the formation of the Spemann organizer.

3.4.1. Functional conservation of Wnt and Nodal response elements in organizer promoters

The Wnt and Nodal pathways cooperate to activate transcription of the organizer genes *Gsc*, *Cer*, and *Chd* through adjacent Wnt and Nodal responsive cis-regulatory elements present in the proximal promoters close to the start site of transcription (this study; Watabe et al., 1995; Yamamoto et al., 2003). Functional conservation of these promoters can be observed in the sequence of the response elements, the proximity of the two elements, and their distance from the start site of transcription. The Sia/Twn

response is mediated by defined P3 elements present in each of the promoters (this study; Bae et al., 2011; Laurent et al., 1997; Watabe et al., 1995; Yamamoto et al., 2003). Elements mediating the FoxH1-dependent response to Nodal signals have been identified in close proximity to the Sia/Twn elements of each promoter, but are less conserved in sequence (Fig. 3.3); (Labbe et al., 1998; Zhou et al., 1998). For *Gsc*, *Cer* and *Chd*, the two response elements are in close proximity, and are separated by no more than 42 bp (Fig. 3.3C) (Watabe et al., 1995; Yamamoto et al., 2003). And in each case, the pair of response elements has a strikingly similar location within 250 bp of the start site of transcription (–226 for *Gsc*, –216 for *Cer*, and –211 for *Chd*) (this study; Watabe et al., 1995; Yamamoto et al., 2003). The similar features of three organizer gene promoters strongly argue for functional conservation in mediating the response to Wnt and Nodal signaling inputs.

The close proximity of the *Gsc*, *Cer*, and *Chd* regulatory elements to the start site of transcription suggests that the Wnt and Nodal effectors and their coactivators may directly interact with the basal transcriptional machinery. In contrast to distal regulatory elements, the proximal elements found in the organizer genes may not require extensive DNA looping or the recruitment of the enzymatic machinery that brings distal effectors in contact with the basal transcriptional machinery (reviewed in Levine, 2010). The close proximity of the Wnt and Nodal response elements to the TATA element (less 200 bp) may allow an immediate interaction of the activating effector complex with the basal transcriptional machinery, resulting in a rapid and robust activation of organizer gene expression upon receipt of Wnt and Nodal inputs. Thus, the functional organization of these promoters may facilitate a rapid transcriptional response, which is essential for the spatially and temporally specific onset of organizer gene expression in the gastrula. This

promoter organization may provide regulatory robustness during a critical developmental period, when even small variation in the timing or level of organizer gene function would result in axial patterning defects (reviewed in Heasman, 2006).

3.4.2. Wnt and Nodal effectors synergistically activate organizer gene transcription

At enhancer regions, multiple bound transcription factors may interact to synergistically activate a strong transcriptional output. A number of mechanisms may account for synergy, including cooperative binding to regulatory elements, cooperative recruitment of coactivators, as well as alterations in DNA conformation or nucleosome deposition (reviewed in Levine, 2010). The synergy in activation of *Gsc*, *Cer*, and *Chd* may reflect one or several of these mechanisms. While it remains unclear whether cooperative binding is occurring among the Wnt and Nodal effectors, our data clearly demonstrate that the steady state binding of transcriptional effectors is increased when Wnt and Nodal pathway effectors occupy these promoters together (Fig. 3.5). This suggests that the presence of Sia/Twn with FoxH1 and Smad2/3 at organizer gene promoters facilitates enhanced occupancy, which is suggestive of cooperative binding.

The common coactivator and lysine acetyltransferase, p300, is recruited to organizer gene promoters in response to both the Wnt and Nodal pathways (Fig. 3.6D-F). The role that p300 plays in the synergistic transcription of organizer genes in response to Wnt and Nodal is not yet understood. Our results demonstrate a requirement for p300 activity in the expression of a *Gsc* reporter, as well as increased occupancy of p300 in the presence of Sia/Twn or Nodal (Fig. 53.6. However, we do not observe further enhancement of p300 occupancy in response to the combination of Wnt and Nodal (data not shown). Perhaps p300 provides a permissive function for

transcription, while other recruited coactivators provide an activating function (reviewed in Bedford et al., 2010). Similarly, p300 could be acting as a scaffolding protein, either stabilizing a transcriptional complex of both Wnt and Nodal effectors, or allowing effectors to interact with other coactivators and/or the basal transcriptional machinery (reviewed in Bedford et al., 2010). p300 has also been shown to acetylate transcription factors and histones (reviewed in Bedford et al., 2010); the combined effects of Wnt and Nodal inputs could enhance p300 enzymatic activity, resulting in more extensive modification of local histones or transcription factors and increased transcription. In the context of organizer gene expression, changes in histone H3K9/14 or H4K5/8/12/16 acetylation have not been observed in response to Wnt or Nodal signals (data not shown). However, p300 is also known to modify other lysine residues in histone tails, such as H3K18/27 (Jin et al., 2011), as well as transcription factors (reviewed in Bedford et al., 2010). Activated Smad2/3 can be acetylated by p300, which increases transcriptional activity (Inoue et al., 2007; Ross et al., 2006; Tu and Luo, 2007). Work in our lab suggests that Sia can be acetylated (data not shown), however, it is unclear what role acetylation might play in Sia-dependent transcription, or whether other Nodal or Wnt effectors might be acetylated in a signal-dependent manner.

The protein composition of the transcriptional complex formed at organizer gene promoters remains to be fully characterized. It will be interesting to examine the role of the interactions of the Wnt and Nodal effectors with other transcriptional regulators of organizer gene expression, such as Xlim1, Mix.1 and Xotx2, which have been shown to cooperatively activate *Cer* transcription together with Sia (Yamamoto et al., 2003). Furthermore, examination of additional histone modifications, such as methylation, and the recruitment of other key coregulators such as SWI/SNF, will be important for fully

defining the molecular mechanisms of organizer gene transcription.

3.4.3. Transcriptional integration of inductive signals in development

In the *Xenopus* gastrula, Wnt and Nodal signaling inputs are integrated at the level of organizer gene promoters (Watabe et al., 1995; Yamamoto et al., 2003). The pathway effectors form an activating complex that enhances transcriptional output, leading to specific gene expression responses within a distinct region of the embryo. Establishment of this transcriptional domain is essential for the formation of the Spemann organizer, which is required for patterning of the embryonic axes (reviewed in De Robertis et al., 2000). Such integration of multiple signaling inputs at developmental enhancers represents a frequently used regulatory strategy in embryogenesis (reviewed in Levine, 2010). In both invertebrate and vertebrate embryos, the complex positional and temporal information provided by inductive signals is received and integrated by combinations of promoter elements, resulting in specific gene expression responses (reviewed in Levine, 2010).

A well-defined example of the transcriptional mechanism we propose is found in *Drosophila* eye development. In the fly eye, *dPax2* is both necessary and sufficient for specification of cone cell fate (Flores et al., 2000). *In vivo* experiments identified responsive elements within the eye enhancer of the *dPax2* gene, revealing that the expression pattern relies on a combination of signaling inputs from a receptor tyrosine kinase called *Sevenless*, the EGF and Notch pathways (Flores et al., 2000). These signals are received in a particular order during development to ensure proper temporal and spatial expression of *dPax2*, the central regulator of cone cell formation (Flores et al., 2000). Studies of binding site organization within the eye-specific enhancer of *dPax2*

provided a number of mechanistic insights (Swanson et al., 2010). For example, the sequences separating the *Sevenless*, EGF, and Notch effector binding sites are essential for proper gene expression and likely contain binding sites for yet to be identified transcriptional regulators (Swanson et al., 2010). In some areas of the enhancer, the spacing between transcription factor binding sites was shown to be essential, as an alteration of this spacing disrupted proper cone cell gene expression (Swanson et al., 2010). And, perhaps most interesting, rearrangement of the elements within the enhancer region led to ectopic expression of *dPax2* in non-cone cells, demonstrating that the *dPax2* enhancer structure confers spatial control of gene expression (Swanson et al., 2010). It remains to be determined whether the spatial arrangement of the Nodal and Wnt response elements of *Gsc*, *Cer*, or *Chd* is important for proper gene expression within the Spemann organizer domain. Furthermore, whether sequences between the Wnt and Nodal response elements are important for additional regulatory inputs has not been examined. Our studies demonstrate that the functional organization of the organizer gene promoters integrates multiple signaling inputs, a gene regulatory strategy that has been utilized in the development of multiple lineages in many systems (reviewed in Levine, 2010).

3.4.4. Conserved and non-conserved aspects of organizer gene regulation

In this work we define a molecular mechanism for the transcriptional integration of Wnt and Nodal signals at organizer gene promoters in the *Xenopus* gastrula. We further propose that this mechanism is likely utilized in multiple vertebrate species to establish the organizer transcriptional domain. Support for the conservation of this mechanism across vertebrates comes from regulatory similarities in organizer formation,

organizer gene expression and organizer gene promoter structure (reviewed in De Robertis et al., 2000). Wnt and Nodal signals are essential for organizer gene expression and organizer formation in *Xenopus*, zebrafish, chick and mouse (Boettger et al., 2001; Conlon et al., 1994; De Robertis et al., 2000; Liu et al., 1999). The functional organization of organizer gene promoters is also conserved to an extent. Most strikingly in the case of *Gsc*, highly conserved DE and PE elements are present in the *Xenopus,* zebrafish, chick, mouse, and human genes (Bae et al., 2011; Watabe et al., 1995). For *Cer*, conserved response elements are present in *Xenopus*, zebrafish and mouse, but their organization differs among species (Yamamoto et al., 2003). For *Chd*, the available genomic information is insufficient for a conclusive comparison. The effectors of Nodal signaling, FoxH1 and Smad2/3, are also utilized in the control of organizer gene transcription in these vertebrate systems (Boettger et al., 2001; Conlon et al., 1994; Hoodless et al., 2001; Nomura and Li, 1998; Waldrip et al., 1998; Weinstein et al., 1998; Zhou et al., 1993).

In contrast to these many conserved features, Siamois and Twin are only found in amphibian species, and not in other vertebrates. Given that Wnt inputs and the PE element are conserved across species (Bae et al., 2011; De Robertis et al., 2000; Heasman, 2006; Watabe et al., 1995), it is likely that functional homologs of Sia/Twn that mediate Wnt-dependent transcriptional activation via the PE exist in other vertebrate species . Alternatively, Sia/Twn may serve a regulatory function that is unique to organizer gene regulation in *Xenopus*; if this is the case, conservation of the PE may reflect distinct regulatory requirements among species. It should be noted that Sia/Twn are not the only species-specific regulators of organizer formation. In zebrafish, the transcriptional repressor *bozozok* is a direct target of the Wnt pathway, is expressed

very early in organizer formation, and is essential for organizer gene expression and organizer formation (Fekany et al., 1999; Koos and Ho, 1999; Shimizu et al., 2000; Solnica-Krezel and Driever, 2001; Yamanaka et al., 1998). However, as is the case for Sia/Twn, no vertebrate orthologs of *bozozok* have been identified. Whether functional homologs of Sia/Twn and *bozozok* exist in other species or whether these factors carry out species-specific regulatory functions remains to be seen. Given the dramatically different sizes and developmental rates for vertebrate embryos, and the non-autonomous function of the organizer, temporal and spatial constraints for organizer formation may differ among species. The non-conserved regulatory components found in *Xenopus* and zebrafish may be necessary for the unique regulatory demands of organizer formation in distinct species.

A number of important regulatory aspects of organizer gene expression continue to be undefined. The full composition and structure of the activating protein complex that forms at organizer gene promoters remains to be determined. How the Wnt and Nodal pathway effectors interact physically, what modifications occur in response to co-factor recruitment, and how together these result in enhanced, yet spatially restricted transcriptional output are important mechanistic questions to answer. Our results offer a molecular mechanism for the initiation of organizer gene expression in a spatially and temporally precise manner. However, organizer gene expression is a dynamic process with changing regulatory inputs as development proceeds. Within 60 minutes of the initiation of organizer gene expression it is likely that promoter occupancy and regulatory complex formation changes dramatically as the initiation phase gives way to maintenance phase or cell lineage specification. Whether the mechanism we propose for the initiation of organizer gene expression is broadly applicable to the many known

organizer genes, and across species as well, will require genome wide analyses of effector occupancy, co-regulator recruitment, and chromatin modification in several vertebrate species. Ongoing studies such as these will provide profound mechanistic insight at the interface of transcriptional control and embryonic pattern formation.

Chapter 4 : Identification of the Functional Domains of Siamois and Twin

4.1. Summary

During early gastrulation, formation of the Spemann organizer is due, in part, to the activity of two homeodomain proteins, Siamois (Sia) and Twin (Twn). Sia and Twn play an essential role in organizer formation downstream of the Wnt signaling pathway by regulating a number of organizer genes, yet little is known of their functional domains. The N-terminal regions of the proteins have high homology in three regions, termed the A, B and C domains. Here, we identify the N-terminal region of Sia and Twn as being essential for transactivation function and secondary axis induction. We find that Sia contains one transactivation domain, within the conserved B region of the protein, while Twn has two transactivation domains, one each within the A and B domains, respectively. A comparison of the inactive A domain of Sia and the active A domain of Twn reveals a single amino acid difference, which, when changed from a serine to a tyrosine, restores transactivation function to the Sia A domain. Lastly, we find that three conserved lysine residues within the C domain of Sia and Twn contribute to transcriptional activity, possibly by acting as a substrate for post-translational modification. Taken together, we identify important regulatory domains within the transcriptional activators Sia and Twn, essential factors downstream of Wnt signaling in the formation of the organizer.

4.2. Introduction

During early embryogenesis, the Spemann organizer patterns the three germ layers and regulates formation of the embryonic axes (reviewed in Harland and Gerhart,

1997). Shortly after fertilization, the Wnt pathway becomes activated on the future dorsal side of the embryo, initiating a cascade of signaling which contributes significantly to organizer formation (reviewed in Heasman, 2006). In response to this early Wnt pathway, two transcriptional activators are expressed, Siamois (Sia) and Twin (Twn) (Brannon and Kimelman, 1996; Carnac et al., 1996; Crease et al., 1998; Fan et al., 1998; Laurent et al., 1997; Nishita et al., 2000). Sia and Twn are direct targets of the Wnt signaling pathway and are required downstream of Wnt in organizer formation (Bae et al., 2011; Brannon and Kimelman, 1996; Carnac et al., 1996; Crease et al., 1998; Fan et al., 1998; Ishibashi et al., 2008; Laurent et al., 1997; Nishita et al., 2000). Sia and Twn were identified in screens to identify the molecular basis of organizer formation (Laurent et al., 1997; Lemaire et al., 1995). The Sia homeodomain was found to be most similar to the Mix family of proteins, but Sia lacked other identifiable domains (Lemaire et al., 1995). Upon isolation of Twn, several conserved and potentially important domains were identified in Sia and Twn, with 88% sequence similarity in the homeodomain and highly conserved sequence in three other regions, termed the A, B, and C domains, N-terminal to the homeodomain (Fig. 4.1 and (Laurent et al., 1997).

Sia and Twn exhibit an 88% homology across the entire homeodomain; however, there is 100% homology within the third helix of the homeodomain, which is important in forming contacts with DNA, suggesting that Sia/Twn may share transcriptional targets (Kessler, 1997; Wilson et al., 1995). Indeed, further work has revealed that Sia and Twn are redundant factors that, together, are required for organizer formation downstream of the Wnt signaling pathway (Bae et al., 2011; Ishibashi et al., 2008). Given their importance in organizer formation, identification of functional domains of Sia/Twn may reveal important co-factor recruitment regions or help identify proteins with homologous

Figure 4.1 Siamois and Twin Conserved Domains

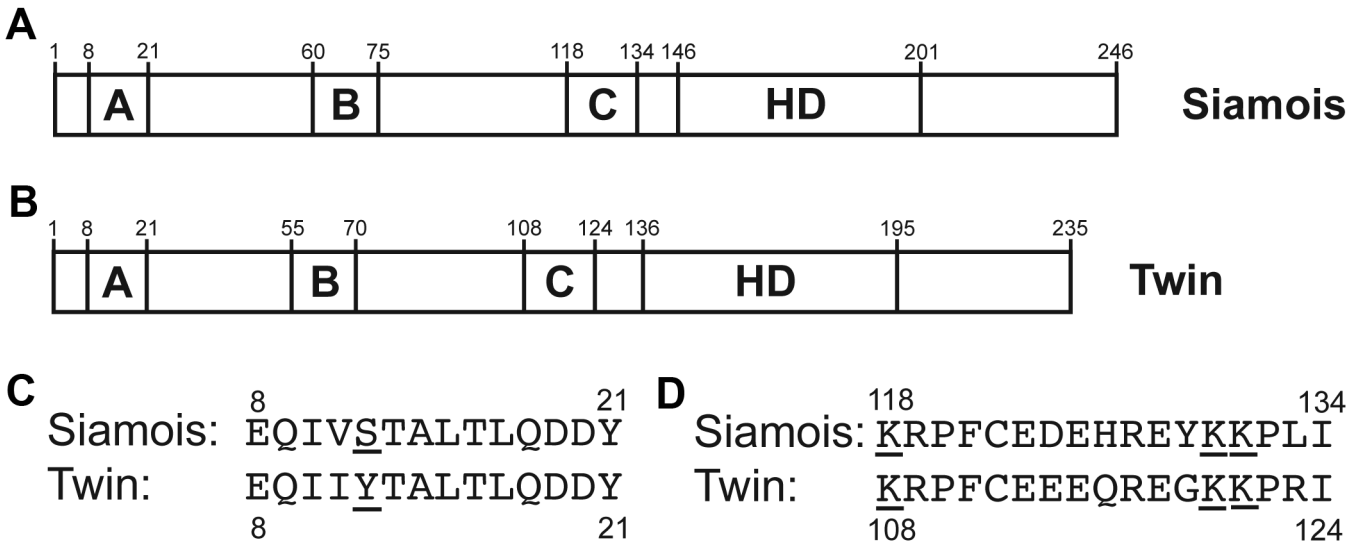


Figure 4.1: Siamois and Twin Conserved Domains

(A-B) Schematic of the Siamois and Twin proteins, with the location of the conserved A, B, and C domains and the homeodomain (HD) indicated. Amino acid number is indicated on the top of the schematic. (C) Amino acid sequence for the A domains of Siamois and Twin, with the twelfth amino acid underlined. Amino acid number is indicated on the first and last residues. (D) Amino acid sequence of the C domains of Siamois and Twin, with the conserved lysine residues underlined. Amino acid number is indicated on the first and last residues.

functional regions in other vertebrates. To this end, we examined the functions of the N terminal regions, containing the A and B conserved domains of Sia and Twn and found these domains to be essential for transcriptional activity and ectopic axis formation. Further deletion of these regions revealed that the single activation domain of Sia lies within the B domain, while Twn contains two activation domains, within the A domain and the B domain. A comparison of the inactive Sia A domain to the active Twn A domain revealed a single amino acid difference at position 12, a serine in Sia, a tyrosine in Twn. Substitution of a tyrosine for the serine at position 12 in Sia was sufficient to confer transcriptional activity and ectopic axis forming activity to an otherwise inactive form of Sia. Lastly, mutation of conserved lysine residues within the C domains of Sia and Twn resulted in a decrease in transcriptional activity, suggesting that these residues may be substrates for post-translational modification of Sia/Twn, which could modulate protein stability or activity. Taken together, we have identified potential regulatory domains of Sia and Twn that may contribute to Sia/Twn activity during early embryogenesis.

4.3. Results

4.3.1. Identification of the activation domains of Siamois and Twin

To identify the transactivation domain of Sia, regions of Sia were fused to the GAL4 DNA binding domain (GAL4 DBD) and these fusion constructs were co-expressed with the 5XUAS luciferase reporter to test for transcriptional activity. The GAL4 DBD alone had little effect on transcription, while Gal4-Sia, containing the full length Sia protein including the homeodomain, activated luciferase expression approximately 55-fold. Subdividing the Sia protein into an N-terminal region (amino acids 1-133, including the A, B, and C

domains) and a C terminal region (amino acids 131-246, including the homeodomain) revealed that the N terminal domain retained transcriptional activity (150-fold), while the C terminus had a much lower level of activity (7-fold) (Fig. 4.2A). The fusion of Gal4-Sia1-75 activated transcription nearly 400-fold (Fig. 4.2A), suggesting that the transactivation domain of Sia lies within the first 75 amino acids. This region contains both the A and B domains, which were separated and tested for transcriptional activity. Gal4-Sia1-39, which contains the A domain had little activity (1.4-fold) while Gal4-Sia40-75, which contains the B domain, retained activity (132-fold) (Fig. 4.2A). Equal expression of constructs was confirmed by western blot analysis on embryonic extracts (data not shown). These results suggest that the B domain of Sia acts as the transactivation domain. The high level of homology between the Sia and Twn B regions suggests that the Twn B region may also act as a transactivation domain.

In order to determine whether the Sia and Twn B domains are transactivation domains, we created constructs of Sia and Twn that deleted the putative activation domains and assessed their abilities to activate transcription of the *Gsc* luciferase reporter (-226 *Gsc* luciferase). While full length Sia and Twn activate this reporter (17.9-fold and 19.4-fold, respectively), SiaΔ75 and TwnΔ70, which lack the A and B domains, fail to activate transcription of the reporter (1.5-fold or 1.2 fold, respectively) (Fig. 4.2B), confirming that the activation domains of Sia and Twn lie within these N terminal domains. Elimination of the B domain of Sia (SiaΔ40-75) prevented transcriptional activation (1.6-fold), while Sia lacking the A domain (SiaΔ39) was able to activate transcription (6.5-fold), although at a lower level than full length Sia protein (Fig. 4.2B). Taken together, these results suggest that the Sia B domain acts as the transactivation domain, as Sia lacking this domain is unable to activate a known target reporter.

Figure 4.2 Identification of the activation domains of Siamois and Twin

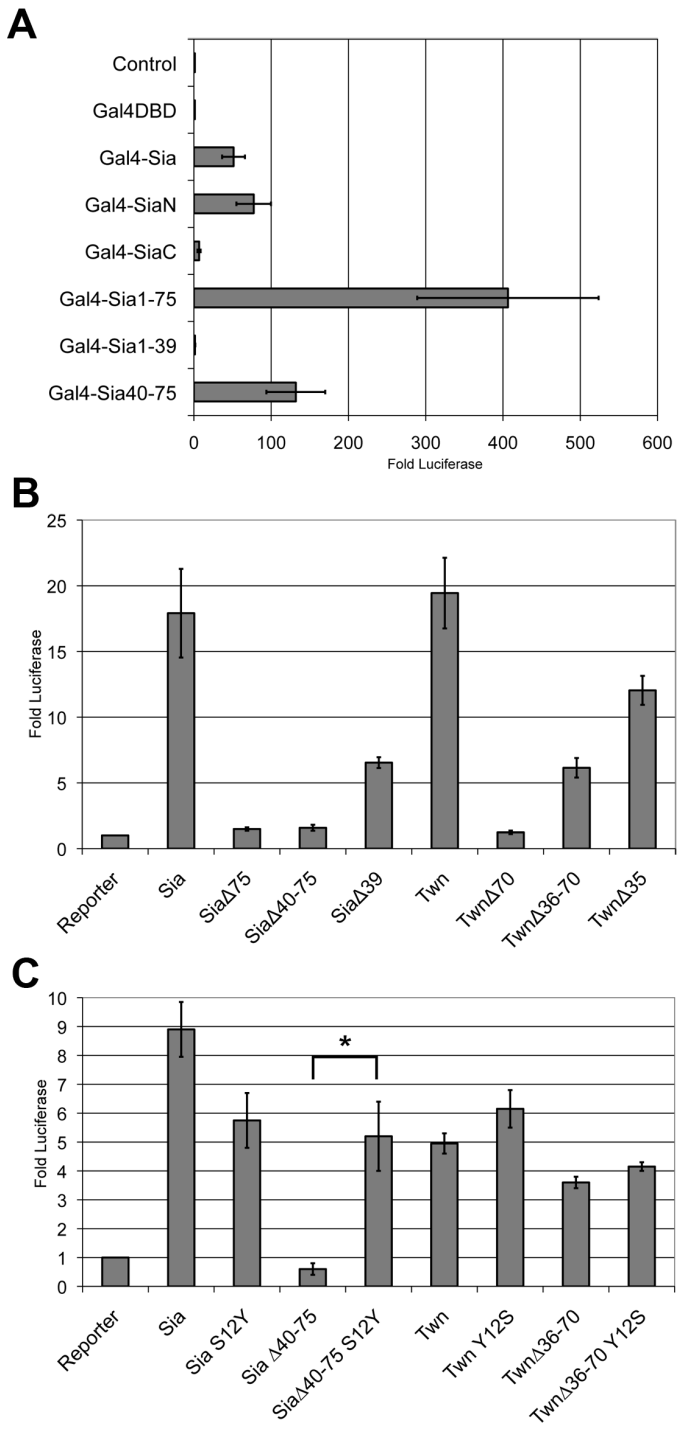


Figure 4.2 Identification of the activation domains of Siamois and Twin

(A) To identify the Sia transactivation domain, one-cell stage embryos were injected with 50pg of Gal4 DNA binding domain (Gal4DBD) or regions of Sia fused to the Gal4DBD. At the two-cell stage plasmid encoding 5X-UAS-Luciferase reporter (100pg) was injected together with CMV-Renilla Luciferase (10pg). (B) To determine the activation domains of Sia and Twn, one-cell stage embryos were injected with 50pg of full length Sia, SiaΔ75 (a deletion of the A and B domains), SiaΔ40-75 (a deletion of the B domain), SiaΔ39 (a deletion of the A domain), full length Twn, TwnΔ70 (a deletion of the A and B domains), TwnΔ36-70 (a deletion of the B domain) or TwnΔ35 (a deletion of the A domain). At the two-cell stage plasmid encoding -226 *Gsc*-Luciferase reporter (100pg) was injected together with CMV-Renilla Luciferase (10pg). (C) To determine whether an amino acid difference in Sia may contribute to transactivation, one-cell stage embryos were injected with 50pg of full length Sia, SiaS12Y, SiaΔ40-75, SiaΔ40-75 S12Y, full length Twn, TwnY12S, TwnΔ36-70 or Twn ΔΔ36-70 Y12S. At the two-cell stage, plasmid encoding -226 *Gsc*-Luciferase reporter (100pg) was injected together with CMV-Renilla Luciferase (10pg). There was a significant increase in transcriptional activity between SiaΔ40-75, which had little activity, and Sia40-75 S12Y. * indicates a p-value <0.05. For these experiments, animal explants prepared at the blastula stage were assayed for luciferase activity at the midgastrula stage. Values shown are normalized to Renilla luciferase activity, and represent fold activation of basal reporter activity in the absence of injected mRNAs. The mean increase in luciferase activity and standard error for four independent experiments is presented.

The high level of conservation between the Sia and Twn B domains predicts that the Twn B domain may act as the transactivation domain of Twn. Interestingly, deletion of either the A or B domains of Twn (TwnΔ35, TwnΔ36-70) did not eliminate the transcriptional activity of Twn. While both TwnΔ35 and TwnΔ36-70 activated transcription (12.0-fold and 6.2-fold, respectively), it was not as high as the activity of full length Twn (19.4-fold) (Fig. 4.2B). This suggests that Twn, unlike Sia, may contain two activation domains, one within the A domain and one within the B domain. The lower transcriptional activity of TwnΔ35 and TwnΔ36-70 suggests that optimal function of Twn may require both domains.

Both Sia and Twn were identified in screens to identify factors involved in organizer formation; as such, overexpression of Sia or Twn in a ventral blastomere results in formation of an ectopic axis (Laurent et al., 1997; Lemaire et al., 1995). Sia or Twn expression at lower doses (1-5pg mRNA) induces a partial axis, consisting largely of trunk tissue, and lacking more anterior tissue (Bae et al., 2011). Ventral expression of Sia or Twn at higher doses (10-30pg mRNA) induces complete ectopic axes, consisting of both trunk and anterior tissue, including notochord, eye and cement gland (Bae et al., 2011). Sia was identified as a transcriptional activator, and repression of Sia target genes by expression of an engrailed-Sia fusion protein prevents axis formation (Fan and Sokol, 1997; Kessler, 1997). We therefore thought it likely that both Sia and Twn would require the activation domain to induce ectopic axis formation.

As previously reported, full length Sia induced a complete secondary axis in nearly all (98%) injected embryos (Table 4.1). Expression of the N-terminal deletion of Sia, lacking both the A and B domains (SiaΔ75), was unable to induce a secondary axis even at high doses (data not shown and Table 4.1). Ventral expression of Sia lacking the

Table 4.1 Frequencies of axis induction by Sia and Twn deletion mutants.

		Ectopic Axis Induction			
mRNA injected	**N=**	**Partial**	**% Partial**	**Complete**	**% Complete**
None	49	0	0%	0	0%
Siamois	42	0	0%	41	98%
SiaΔ75	41	0	0%	0	0%
SiaΔ40-75	50	0	0%	0	0%
SiaΔ39	43	13	30%	15	35%
Twin	40	0	0%	39	98%
TwnΔ70	46	0	0%	0	0%
TwnΔ36-70	48	24	50%	23	48%
TwnΔ35	45	16	36%	22	49%

At the four cell stage, a single ventral blastomere was injected with 10pg of the indicated mRNA. Embryos were scored for ectopic axis induction at the neurula stage. The partial axis class contained ectopic trunk tissue while the complex axis class contained ectopic trunk and head structures, including the anterior structures of the eye and cement gland. N indicates the total number of embryos analyzed for each experimental condition. Data is presented as the number of embryos observed with partial or complete ectopic axes as well as the percentage of embryos observed with partial or complete ectopic axes.

B domain, which was identified earlier as the transactivation domain, did not result in ectopic axis formation, suggesting that the transcriptional activation function of Sia is required for the formation of an ectopic axis. SiaΔ39, which lacks the A domain, induced ectopic axis formation in 65% of embryos, with 30% of embryos forming partial secondary axes and 35% of embryos forming complete secondary axes (Table 4.1). These results reveal that SiaΔ39 is not as active as full length Sia, suggesting that the Sia A domain may contribute to the full activity of Sia. Taken together, these data confirm that the Sia B domain acts as the activation domain within the Sia protein, and this domain is necessary for Sia-mediated ectopic axis formation.

We next tested the ability of the Twn deletion constructs to induce ectopic axes. As previously reported, Twn was able to induce a complete secondary axis in 98% of embryos, and a deletion of the A and B domains of Twn (TwnΔ70) induced ectopic axis formation in 0% of embryos (Table 4.1). Ventral expression of Twn lacking the A domain(TwnΔ35) led to formation of a partial secondary axis in 36% of embryos and a complete secondary axis in 49% of embryos. Ventral expression of Twn lacking the B domain (TwnΔ36-70) led to formation of a partial secondary axis in 50% of embryos and a complete secondary axis in 48% of embryos (Table 4.1). The frequency of complete axis formation with TwnΔ35 or TwnΔ36-70 was lower than full length Twn (49%, 48% and 98%, respectively), suggesting that the A and B domains together may contribute to optimal secondary axis induction. Taken together, we have found that Sia contains one transactivation domain within the conserved B region of the protein, while Twn contains two transactivation domains within both the A and B region.

Given the high levels of conservation between Sia and Twn, especially within the A and B domains (illustrated in Fig. 4.1A-C, and (Laurent et al., 1997), we found these results

to be quite curious. Both the Sia and Twn B domains are able to confer transcriptional activity, as both SiaΔ39 and TwnΔ35 activated the *Gsc* luciferase reporters. However, while the A domain of Twn is sufficient to confer transcriptional activity, the A domain of Sia is not. This difference could be due to the amino acids between the A and B domains in Sia/Twn, but deletion of this region in either protein (SiaΔ22-59, TwnΔ22-54) had little effect on secondary axis induction (Table 4.2). Alternatively, this difference could be due to an amino acid difference within the A domain at position 12, a serine in Sia and a tyrosine in Twn (Fig. 4.1C). We sought to determine whether this amino acid difference had any effect on the transcriptional activity of Sia by substituting a tyrosine for the serine in both full length Sia (SiaS12Y) as well as Sia lacking the B domain (SiaΔ40-75 S12Y). We also made the converse change in Twn, substituting a serine for the tyrosine at position 12 in the full-length protein (TwnY12S) and in the B domain deletion of Twn (TwnΔ36-70 Y12S). These constructs were tested for their ability to activate transcription of the *Gsc* luciferase reporter as well as their ability to induce the formation of a secondary axis when expressed in a ventral blastomere. While the B deletion of Sia, SiaΔ40-75, was unable to activate transcription from the *Gsc* luciferase reporter (Fig. 4.2B,C), SiaΔ40-75 S12Y had significant transcriptional activity (Fig. 4.2C). Similarly, SiaΔ40-75 S12Y induced formation of a partial axis in 59% of embryos and a complete secondary axes in 31% of embryos, suggesting that a single amino acid substitution at position 12 of the Sia protein renders the A domain unable to activate transcription. The amino acid change in the full length Sia protein (SiaS12Y) did not confer additional transactivation or axis inducing function (Fig. 4.1C, Table 4.2), and the converse changes in Twn (TwnY12S or TwnΔ36-70 Y12S) did not have a significant effect on transcriptional activity or axis induction (Fig. 4.1C, Table 4.2), suggesting that

Table 4.2 Axis induction by Sia and Twn A domain substitution mutants.

		Ectopic Axis Induction			
mRNA injected	**N=**	**Partial**	**% Partial**	**Complete**	**% Complete**
None	27	0	0%	0	0%
Siamois	35	12	34%	20	57%
SiaS12Y	36	11	31%	18	50%
SiaΔ40-75	32	0	0%	0	0%
SiaΔ40-75 S12Y	32	19	59%	10	31%
SiaΔ22-59	37	15	41%	19	51%
Twin	42	8	19%	34	81%
TwnY12S	34	8	24%	25	74%
TwnΔ36-70	41	12	29%	25	61%
TwnΔ36-70 Y12S	34	8	24%	24	71%
TwnΔ22-54	31	15	48%	10	32%

At the four cell stage, a single ventral blastomere was injected with 10pg of the indicated mRNA. Embryos were scored for ectopic axis induction at the neurula stage. The partial axis class contained ectopic trunk tissue while the complex axis class contained ectopic trunk and head structures, including the anterior structures of the eye and cement gland. N indicates the total number of embryos analyzed for each experimental condition. Data is presented as the number of embryos observed with partial or complete ectopic axes as well as the percentage of embryos observed with partial or complete ectopic axes.

these residues in Twn are not necessary for Twn mediated transcriptional activation. In conclusion, the transactivation domain of Sia lies within the conserved B region of the protein; a single amino acid change can change the normally inactive Sia A domain into an active transactivation domain, able to induce transcription and secondary axis induction.

4.3.2. Function of the C domain in Siamois and Twin

The identification of the A and B domains of Sia and Twn as potential activation domains suggested that perhaps the other highly conserved domain of Sia and Twn, the C domain, might play some role in Sia/Twn function. Deletion of the C domain had no effect on transcriptional activity of Sia (data not shown), which suggested that the C domain may have another role in the modulation of Sia/Twn activity. When expressed as a tagged form in embryos, both Sia and Twn ran nearly 10kDa larger than their predicted size (with the myc tag, Sia/Twn should be about 36kDa, but generally ran larger than 45kDa (see Fig. 4.3A and data not shown). However, *in vitro* transcribed and translated Sia protein generally ran at the predicted size, around 30kDa (data not shown), suggesting that Sia and Twn may be post translationally modified in the embryo, leading to a larger overall protein size. Incubation of Sia protein with PCAF, a protein acetyltransferase, and a radiolabelled acetyl donor, led to acetylation of Sia, primarily within the C domain (data not shown). We sought to determine whether modifications within the C domain of Sia or Twn play a role in protein function or activity.

Comparison of the C domains of Sia and Twn revealed that both proteins contained three conserved lysine residues within the C domain (Fig. 4.1D). Lysine residues can act as a substrate for several modifications, including methyl and acetyl

Figure 4.3 Brief functional analysis of the C domain of Siamois and Twin

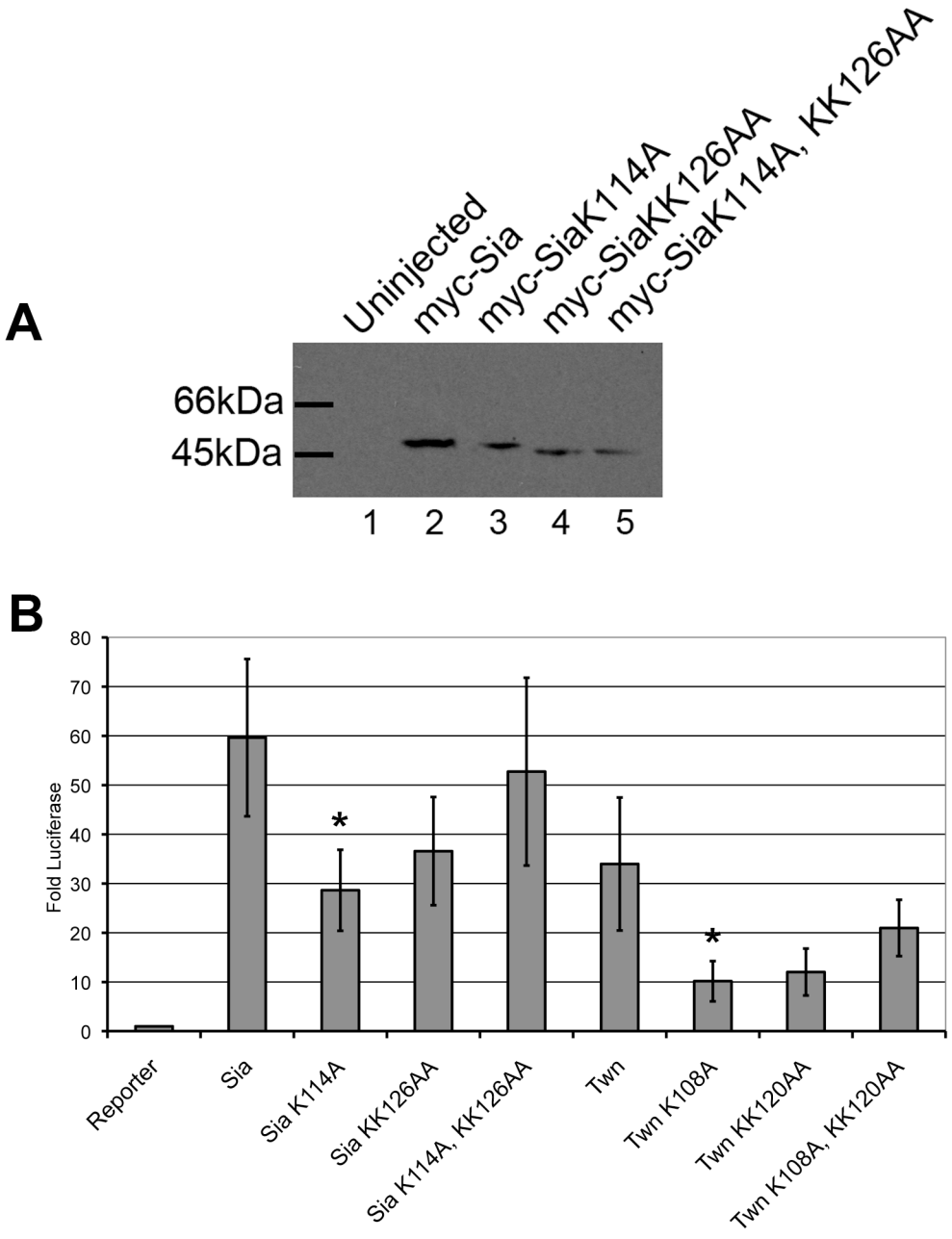


Figure 4.3 Brief functional analysis of the C domain of Siamois and Twin

(A) Extracts prepared from embryos injected with myc-Sia, myc-SiaK114A, myc-SiaKK126AA or myc-SiaK114A, KK126AA mRNA were analyzed by western blotting using an anti-myc antibody to determine protein size. While myc-Sia ran slightly larger than 45kDa, mutation of the lysine domains within Sia resulted in a faster running protein, suggesting that modifications at these residues may cause Sia protein to run larger than expected. (B) To determine whether conserved lysine residues of Sia and Twn may contribute to transcriptional activity, one-cell stage embryos were injected with 50pg of full length Sia; SiaK114A; SiaKK126AA; SiaK114A, KK126AA; full length Twn; Twn K108A; TwnKK120AA; or TwnK108A, KK120AA. At the two-cell stage, plasmid encoding -226 *Gsc*-Luciferase reporter (100pg) was injected together with CMV-Renilla Luciferase (10pg). There was a significant decrease in transcriptional activity between Sia and SiaK114A or between Twn and TwnK108A. * indicates a p-value <0.05. Animal explants prepared at the blastula stage were assayed for luciferase activity at the midgastrula stage. Values shown are normalized to Renilla luciferase activity, and represent fold activation of basal reporter activity in the absence of injected mRNAs. The mean increase in luciferase activity and standard error for three independent experiments is presented.

groups, which can modulate protein activity. We sought to determine whether these conserved lysine residues may play a role in Sia/Twn transcriptional activity. Several constructs of Sia/Twn were made to mutate these conserved lysine residues to alanine. Wild type Sia, as well as SiaK114A, SiaKK126AA and SiaK114A,KK126AA expression in embryonic extracts was analyzed by western blot. While the wild type tagged Sia ran around 45kDa, the SiaKK126AA and SiaK114A,KK126AA were observed to run slightly faster, indicating that these residues might serve as substrates for a post-translational modification of Sia. These constructs were tested for transcriptional activity using the *Gsc* luciferase reporter. Mutation of the first lysine within the C domain of either Sia or Twn to alanine (SiaK114A, TwnK108A) led to a significant decrease in transcriptional activity (Fig. 4.3B), suggesting that modification of this residue might be important in protein function. Mutation of the adjacent pair of lysine residues within the C domain (SiaKK126AA, TwnKK120AA) also resulted in a decrease in transcriptional activity (Fig. 4.3B). Interestingly, mutation of all three residues (SiaK114A, KK126AA; Twn K108A, KK120AA) had little effect on transcriptional activity, as compared to wild type Sia or Twn (Fig. 4.3B). These data suggest that modification of at least one residue within Sia and Twn (SiaK114, TwnK108) may be important in modulating transcriptional activity. Taken together, we find that post translational modifications of Sia/Twn may be important in modulating protein activity. It will be interesting to determine whether these residues are important in the function of Sia and Twn in the embryo and which types of post translational modifications occur *in vivo*.

4.4. Discussion

Sia and Twn are homeodomain proteins that are essential downstream of the

Wnt signaling pathway during Spemann organizer formation. Here, we report an analysis of the conserved A, B and C domains of Sia and Twn to further understand how organizer gene expression is initiated. While the B domain of Sia and Twn activates transcription, only the A domain of Twn has transcriptional activity, indicating that, although highly similar, Sia and Twn do have one indentified difference: Sia contains one activation domain, within the B domain, while both the A and B domains of Twn act as transactivation domains. Further, we find a single amino acid, at position 12 within the Sia A domain, which, when changed from a serine to a tyrosine, confers transactivation function to this domain. We also identify residues within the Sia and Twn C domains that may be important in Sia/Twn function. Mutation of conserved lysine residues within the C domain of Sia or Twn results in a shift in Sia protein size and a decrease in Sia/Twn transcriptional activity. These results suggest that Sia/Twn may be modified post-translationally at these residues. These modifications may modulate Sia/Twn protein function, stability or interactions with co-factors. It will be important to determine whether modifications to these residues contribute to Sia/Twn function in the embryo. Taken together, we identify important regions of the Sia/Twn proteins that activate transcription, and may recruit co-factors and modulate protein activity. These conserved regions of Sia/Twn may help us further identify co-factors and co-regulators that contribute to Sia/Twn function *in vivo*. Further study of these domains may reveal how Sia/Twn act to restrict organizer gene expression to the dorsal domain of the gastrula.

4.4.1. Recruitment of Co-activators by Sia/Twn

Identification of the Sia/Twn activation domain could help to identify potential co-factors utilized by Sia/Twn in organizer gene expression. Sia/Twn are essential for

organizer formation downstream of the Wnt signaling pathway (Bae et al., 2011; Fan and Sokol, 1997; Ishibashi et al., 2008; Kessler, 1997), and have been shown to cooperate synergistically with the Nodal pathway in the activation of the organizer genes *Gsc*, *Cer*, and *Chd* (discussed in Chapter 3). Identification of co-factors recruited in such a scenario might explain the mechanism of synergy and may help identify factors involved in the regulation of organizer gene expression in other vertebrate species. However, the activation domains of Sia/Twn do not contain any identifiable recruitment sequences or regions that might bind to well known co-activators. A candidate approach was taken to identify potential co-factors recruited by Sia/Twn; one such co-factor identified was p300 (see Chapter 3), a histone acetyltransferase that also interacts with Nodal pathway effectors to activate transcription (Inoue et al., 2007; Ross et al., 2006; Tu and Luo, 2007). While p300 activity is required for both Sia/Twn and Nodal activation of target genes (Chapter 3), the exact role of p300 during organizer formation remains to be determined. Whether p300 directly acetylates histone tails, transcription factors such as Sia/Twn, or other proteins involved in transcription remains to be seen. Also, it remains unclear whether Sia/Twn directly recruit p300, or if p300 is part of a larger transcriptional complex that forms at organizer gene promoters. Identification of other recruited co-factors will be important in determining the mechanism of organizer gene expression by Sia/Twn.

4.4.2. The Function of Sia and Twn Conserved Domains

Sia/Twn are essential for the formation of the Spemann organizer (Bae et al., 2011), yet no vertebrate orthologs outside of amphibians have been identified. One possibility is that Sia/Twn carry out amphibian specific aspects of development, and may

be unnecessary in organizer formation in other species. Alternatively, other proteins may function as Sia/Twn, but the sequence of the protein could have diverged significantly. If this is the case, elucidation of functional domains of Sia/Twn could help identify proteins that function in a similar role in other species. Because of the high level of conservation of the Sia/Twn binding site within the *Gsc* proximal element in other vertebrates (Bae et al., 2011), we would predict that a paired type homeodomain protein, like Sia/Twn, would regulate some aspect of *Gsc* expression in other vertebrates. In the sequence of the homeodomain, Sia and Twn are most highly similar to the Mix family of paired-type homeodomain proteins (Laurent et al., 1997; Lemaire et al., 1995), which are downstream effectors of the Nodal signaling pathway (Hart et al., 2005). Mix family members are found in higher vertebrates, including mouse and human, and Mlxl1 has been shown to be involved in early embryogenesis in mouse (Hart et al., 2002). However, Sia/Twn lie downstream of Wnt signaling (Bae et al., 2011; Brannon et al., 1997; Brannon and Kimelman, 1996; Carnac et al., 1996; Crease et al., 1998; Fan et al., 1998; Ishibashi et al., 2008; Kessler, 1997), while Mixl1 is downstream of Nodal signaling (Hart et al., 2005). We find a similar situation in zebrafish, where the homeodomain transcriptional repressor, *bozozok* (Fekany et al., 1999; Koos and Ho, 1999; Yamanaka et al., 1998), is essential for organizer formation and expression of organizer genes such as *gsc* (Shimizu et al., 2000; Solnica-Krezel and Driever, 2001), yet no vertebrate orthologs have been identified. It remains to be determined whether Sia/Twn and *bozozok* represent unique requirements in the development of the frog and the fish, or whether emerging genomic tools will help identify putative orthologs in other model systems.

eidia transactivation domain at some point in evolutionary history, but it does not

appear to do so now. A comparison of the Sia A domain sequence in a closely related amphibian, *Xenopus tropicalis*, reveals conservation of a serine at position 12, suggesting that the A domain of *Xenopus tropicalis* Sia is also inactive. Since Sia and Twn are highly similar in sequence and in function, the pressure to preserve conserved activation domains in these proteins might not be as great. Conversely, Sia and Twn could have both overlapping and distinct functions during organizer formation that may not be discernible with our experimental approaches. Similarly, the Sia A domain may function in an as yet unidentified manner to modulate protein stability, dimerization or other important proteins functions. A similar question persists for the Sia and Twn C domain. The high conservation in this domain suggests it might contribute to protein function; what function, however, remains to be determined. Removal of this domain does not affect transcriptional activity, but elimination of this domain from a Gal4-Sia construct leads to significant enhancement in transcriptional activity (Fig. 4.2A, compare Gal4-SiaN to Gal4-Sia1-75), suggesting that the C domain may function in negatively regulating transcriptional activity. Mutation of one conserved lysine residue within Sia or Twn led to a decrease in transcriptional activity, but mutation of two other lysine residues, or the mutation of combination of all three conserved lysine residues did not have a significant effect on transcription. These results suggest that these residues may play multiple roles in modulating Sia/Twn function and it will be interesting to see how this conserved domain functions in the restriction of organizer gene expression.

In *Xenopus*, *Gsc* is regulated by inputs from both the Wnt and Nodal pathways, through a Nodal responsive Distal Element (DE) and a Wnt responsive Proximal Element (PE). The mouse PE retains Wnt responsiveness in *Xenopus* (Watabe et al., 1995), suggesting that Wnt signals are involved in *Gsc* expression in the mouse. A

search of the mouse genome for homeodomain proteins containing domains similar to the A, B or C domains did not reveal potential candidates. It is likely that combining data from expression profiles, bioinformatics and ChIP sequencing data may reveal promising candidates that could act during organizer formation in other species. The elucidation of the promoter regions of more organizer genes may also reveal transcription factors that fulfill the role of Sia/Twn in other vertebrate species.

Taken together, we have identified important domains within the transcriptional activators Sia and Twn. While further work is needed to elucidate the mechanism of Sia/Twn transcriptional activity, the identification of these domains is important in understanding the function of other proteins containing domains similar to Sia/Twn. A single amino acid within the Sia A domain, which confers transcriptional activity to an otherwise inactive domain, is an important finding and future work will focus on how this residue might contribute to protein structure and co-factor recruitment. Furthermore, future work should also focus on the post-translational modifications of Sia/Twn and their role in modulating protein activity. These modifications could restrict organizer gene expression to the dorsal side of the embryo, merely by limiting the spatial expression of the Sia/Twn modifier. Determining the mechanism of action of Sia/Twn is important in elucidating how transcriptional inputs can result in the restriction of gene expression to the Spemann organizer.

Chapter 5 : Conclusions and Future Directions

5.1. Summary

In this study, we demonstrate the cooperation between Wnt and Nodal inputs at multiple organizer gene promoters. The Wnt pathway effectors, Siamois (Sia) and Twin (Twn) are necessary for organizer formation downstream of maternal Wnt. Sia and Twn mediate this signal via direct binding as homo- or hetero-dimers to the *Goosecoid* (*Gsc*) promoter at a conserved domain. Nodal pathway effectors FoxH1 and Smad2/3 cooperate with Sia/Twn in the activation of several organizer genes. Nodal and Wnt effectors form a transcriptional complex at organizer promoters that results in a synergistic transcriptional response, suggesting a common mechanism for the regulation of genes in the organizer domain. Structure/function analysis of Sia and Twn revealed the function of three domains conserved between the two proteins. This work may provide a model for the integration of signaling inputs at target promoters in other contexts. Elucidating the full nature of the transcriptional complex formed at organizer gene promoters will determine the mechanism behind temporally and spatially restricted gene expression patterns in the early embryo.

5.2. Model for Organizer Gene Transcription

A model for organizer gene expression is shown in Figure 5.1. Activation of the Wnt pathway on the future dorsal side of the embryo results in the expression of Sia and Twn within a restricted domain of the marginal zone (Fig. 5.1A). The pattern of Nodal activation is much more broad, extending across the vegetal hemisphere of the embryo and into the marginal zone (Fig. 5.1B). The activity of these two signals overlaps within

Figure 5.1 Model for the regulation of organizer gene expression

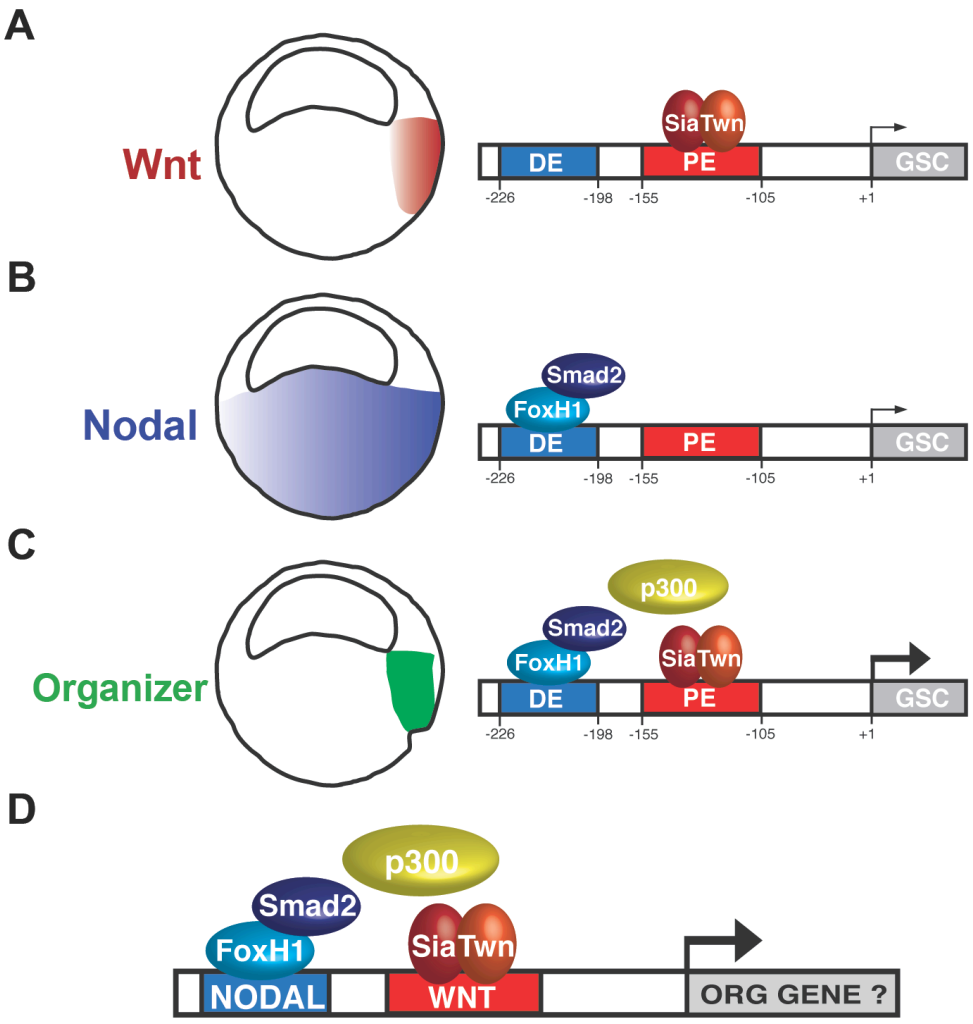


Figure 5.1 Model for the Regulation of Organizer Gene Expression

A simplified schematic showing a bisected, blastula (A-B) and early gastrula (C)stage embryo with animal pole up, future dorsal to the right. (A) Activation of the Maternal Wnt pathway (red) on the future dorsal side of the embryo results in expression of Siamois (Sia) and Twin (Twn), which bind to the Wnt responsive PE of the *Gsc* promoter. Endogenous Sia/Twn alone are not likely to induce high levels of expression of *Gsc*, so a smaller arrow at the start site of transcription indicates a lower level of transcription. (B) Activation of the Nodal pathway (blue) at the blastula stage throughout the vegetal and marginal region of the embryo. Nodal is thought to form a morphogen gradient across the embryo, with highest activity on the dorso-vegetally (darker blue). Activation of the Nodal pathway leads to association of Smad2 (or Smad3, not diagrammed) with FoxH1 at the Nodal responsive element of the *Gsc* promoter. Again, endogenous levels of Nodal alone are not likely to induce high levels of expression of *Gsc*, so a smaller arrow at the start site of transcription indicates a lower level of transcription. (C) The organizer (green) forms at the gastrula stage in an area where the Nodal and Wnt pathways overlap. Expression of *Gsc* is mediated by a transcriptional complex including the Nodal effectors FoxH1 and Smad2/3, the Wnt effectors Sia/Twn and the common co-activator p300. This leads to synergistic activation of transcription (larger arrow at the start site of transcription), resulting in expression of *Gsc* within the organizer domain. (D) Our findings indicate that the combined inputs of Nodal and Wnt may be a common mechanism for the expression of multiple organizer genes, resulting in the synergistic expression of organizer genes in the organizer domain.

the future dorsal domain, an area fated to form the organizer. I hypothesize that the activity of these two inputs cooperates to activate organizer gene expression, resulting in recruitment of co-factors such as p300, and synergistic transcriptional output within this region (Fig. 5.1C).

The identification of three organizer genes that respond synergistically to Sia/Twn and Nodal combined suggests that this may be common mechanism for organizer gene expression. Endogenous inputs from Nodal or Wnt alone may not be sufficient for organizer gene expression, while a combination of effectors from both pathways may be required for robust organizer gene expression within a specific domain (Fig. 5.1D). Future experiments focused on the regulation of the expression of other organizer genes, including identification of regulatory domains and the presence of consensus homeodomain or FoxH1 binding sites, which would reveal whether combined regulation by Wnt and Nodal is a common mechanism. It will be especially interesting to see if this combined regulation translates to mammalian node formation, as *Chordin* and *Noggin* are required for axial patterning in the mouse (Bachiller et al., 2000), and their regulatory domains have yet to be defined. Conserved sequences upstream of the *Noggin* gene contain putative Fox family binding sites, conserved Smad4 binding sites, as well as putative homeodomain sites, suggesting that proteins similar to Sia/Twn and FoxH1 may regulate *Noggin* expression in the mouse. *Chd* regulation is more difficult to discern, as the *Chd* gene is adjacent, and may even overlap with another gene, making identification of potential regulatory sequence more challenging.

Other transcription factors also play important roles in organizer gene expression in concert with Sia/Twn and Nodal. For example, the homeodomain transcription factor Xlim-1 activates expression of *Gsc* at the gastrula stage. However, Xlim-1 occupies a

region nearly 200bp upstream of the *Gsc* DE and PE. Xlim-1 cooperates with Otx2 in the expression of both *Gsc* and *Cer*, suggesting that Xlim-1 and Otx2 mediate organizer gene expression with Sia/Twn and Nodal pathway effectors. It is likely that organizer gene expression requires inputs from multiple transcription factors at multiple enhancer sequences. Activating complexes likely only form within the dorsal domain, while repressive complexes form outside this domain. If antibodies recognizing Sia/Twn, FoxH1, Smad2/3, Xlim-1 and Otx2 were available, it would be interesting to not only dissect the timing of these factors at organizer promoters during late blastula and early gastrula stages, but also to spatially resolve where complexes form within the embryo itself.

Repression of organizer genes outside of the organizer domain is likely to play a role in the restricted expression pattern of organizer genes. For example, FoxH1 acts as a transcriptional repressor, recruiting Groucho co-repressor to target genes in the absence of a Nodal signal (Steiner, Reid and Kessler, unpublished data). Activation of the Nodal pathway within the dorsal domain likely results in a displacement of Groucho co-repressors by phosphorylated Smad2/3, leading to activation of target gene expression. FoxH1 mediated repression of organizer gene expression outside of the organizer domain likely plays an important role in the restricted expression of organizer genes. The Groucho co-repressor, Grg4 occupies the *Gsc* promoter in the absence of Nodal signaling (Reid and Kessler, unpublished data), suggesting that co-repressors may mediate organizer gene repression in the embryo.

Our model is based on data obtained by overexpression of transcripts encoding Wnt and Nodal effectors within the embryo. Overexpression of proteins has been known to cause phenotypes that are not directly related to the normal function of the protein of

interest. While I have taken every care to ensure the data obtained here is consistent with the role of these proteins in the embryo itself, I cannot rule out the possibility that overexpression of factors like Sia/Twn or FoxH1 may force interactions with promoter regions not normally occupied by these factors. In the case of Sia/Twn, a small amount of mRNA (1-5pg) results in ectopic axis formation, but most of my ChIP experiments were performed with moderate amounts of Sia/Twn mRNA (50pg), a dose that results in excessive dorsalization of the embryo (data not shown). At lower doses of Sia/Twn, an association with organizer promoters was observed, but the association was not as robust as with the higher doses. This is a caveat of working with factors that robustly induce organizer formation. While the organizer makes up about 5% of the cells of an embryo, it influences at least half of the cells, resulting in large changes in axis formation in response to more moderate changes in gene expression overall. The ChIP protocol is able to detect endogenous Smad2/3 associated with the organizer promoters (Chapter 3), suggesting that the protocol itself is sensitive enough to detect changes at promoters in small regions of the embryo. The lack of antibodies that detect endogenous Sia/Twn or FoxH1 prevents detection of these proteins at endogenous promoters in an unmanipulated embryos. Similarly, the ability to knockdown gene expression, and not eliminate it genetically, prevents a more complete analysis on the requirement of Wnt and Nodal effectors in organizer formation.

An assembled *Xenopus tropicalis* genome will facilitate the identification of conserved regulatory domains of organizer promoters and will likely lead to the characterization of chromatin marks and changes in those marks throughout early development, as has been undertaken recently. The *Xenopus* embryo remains an ideal model system for the study of early development, as the roles of both the Wnt and Nodal

pathways have been well characterized in the embryo. With large numbers of embryos obtainable on a daily basis, it is likely that *Xenopus* will be used extensively for genomic studies focused on changes to regulatory domains during development. As *Xenopus* has proved to be a useful tool in the study of somatic cell nuclear transfer (SCNT), it is likely that changes in gene expression during early embryogenesis are important in the maintenance of totipotent or pluripotent stem cells.

5.3. Temporal and Spatial Restriction of Organizer Gene Expression

The Wnt pathway was found to require the PE, a region about 50 bases proximal to the DE (Watabe et al., 1995). Deletion of the DE and PE together resulted in a loss of reporter expression within the putative organizer domain, suggesting that both the DE and the PE are necessary for expression of *Gsc* in the embryo (Watabe et al., 1995). Removal of the DE from the *Gsc* promoter restricted luciferase expression to the dorsal-most marginal blastomeres, suggesting that Wnt effectors may act to restrict *Gsc* expression within the early embryo (Watabe et al., 1995). Our work has revealed the importance of Sia/Twn in mediating this Wnt signal, through the conserved P3 site within the PE (see Chapter 2). However, several questions remain. For example, what mediates the Nodal signal and at what time point? Knockdown of FoxH1 expression results in a reduction of *Gsc* expression during early gastrula that recovers to wild type levels at later gastrula stages (Kofron et al., 2004a), suggesting that other factors mediate Nodal signals during late gastrula. At the late gastrula stage, FoxH1 expression is declining (Chen et al., 1996), while the expression of other Nodal effectors, such as Mix.1 or Mixer are maintained (Henry and Melton, 1998; Lemaire et al., 1998), suggesting that multiple Nodal effectors may mediate *Gsc* expression during

development. Similarly, the expression of Sia/Twn declines at late gastrula stages (Lemaire et al., 1995), suggesting that multiple types of transcription factors may regulate the temporal expression of Gsc.

5.4. Further Study of the Regulatory Domain of *Goosecoid*

To test whether the DE and PE are sufficient for organizer domain expression, we designed lacZ constructs with the *Gsc* promoter, containing an intact DE and PE. Reporters were also designed that mutated the DE (termed the M4 mutation, after (Watabe et al., 1995) or the P3 site within the PE (termed the PEX mutation), or containing both the M4 and PEX mutations (Bae et al., 2011; Watabe et al., 1995). Plasmids of these reporters were injected into two dorsal blastomeres of the 4-cell stage embryo and lacZ staining was evaluated at the late gastrula stage (Stage 11). The wild type *Gsc* reporter induced lacZ expression within the dorsal domain, in an area roughly similar to that of endogenous *Gsc* (Fig. 5.2A). Mutation of either the DE or the PE alone had little effect on this expression pattern, although lacZ expression may be reduced in some embryo samples (Fig. 5.2B-C and data not shown). This result suggests that regulatory domains outside of the defined *Gsc* promoter region may be important in regulating repression of *Gsc* expression outside of the organizer domain. Mutation of both the DE and the PE together resulted in a loss of lacZ expression completely (Fig. 5.2D), suggesting that *Gsc* expression is highly dependent on the intact DE and PE. However, regions outside the isolated *Gsc* promoter are likely important in the endogenous regulation of *Gsc*, as the constructs are expressed more broadly in the vegetal and animal regions than *Gsc* itself (Fig. 5.2 and data not shown). These reporters will be useful in generating transgenic *Xenopus* embryos, to determine the full

Figure 5.2 Expression of the *Goosecoid*-lacZ reporter

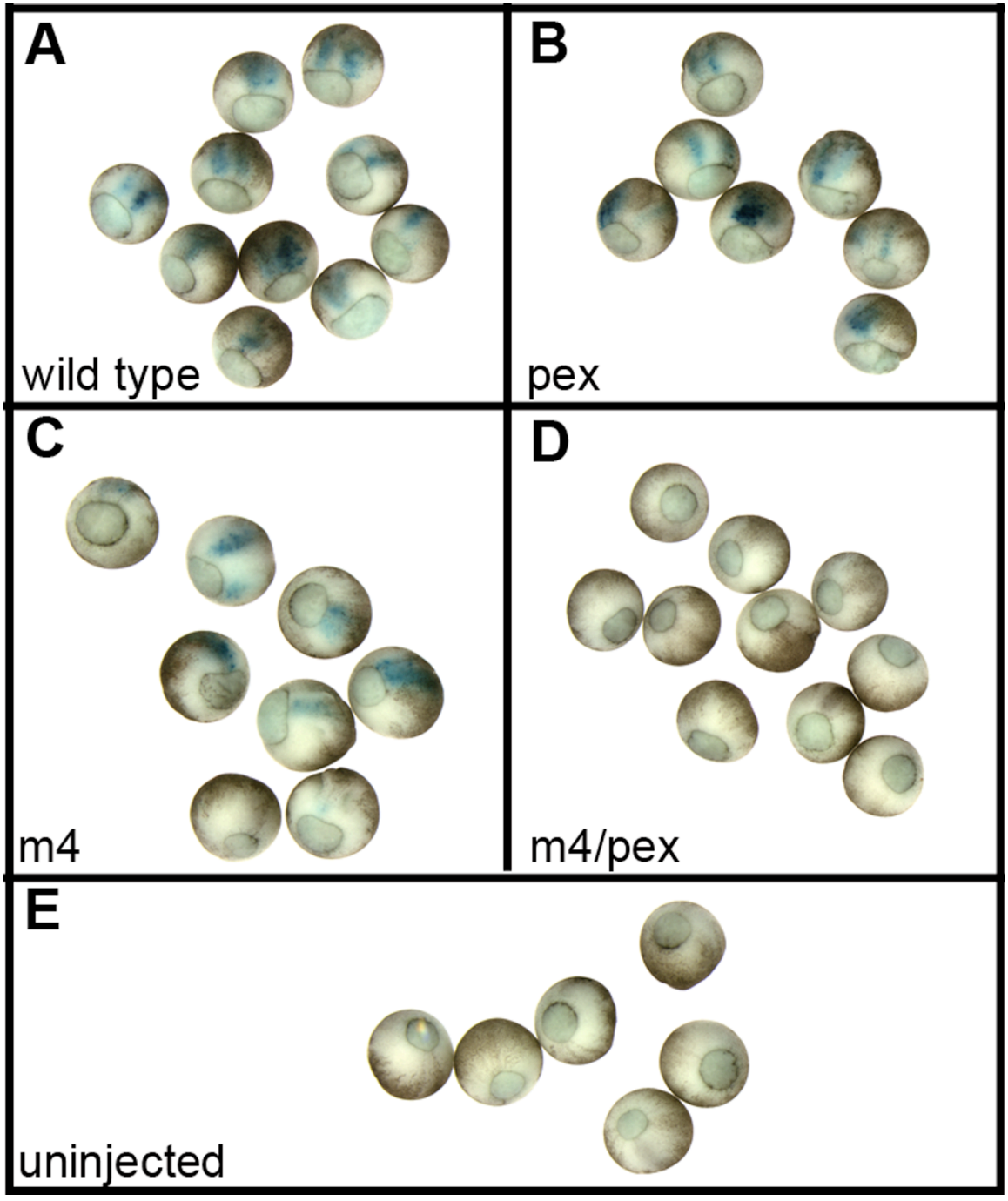


Figure 5.2 Expression of the *Goosecoid*-lacZ reporter

At the four cell stage, two dorsal blastomeres were injected with 500pg of a *Gsc* promoter-lacZ reporter. Embryos were assayed for lacZ expression at the late gastrula stage. (A) Expression of the wild type *Gsc* promoter-lacZ, with intact DE and PE. (B) Expression of the *Gsc* promoter-lacZ reporter containing mutations in the Sia/Twn binding site within the Wnt responsive PE. Expression persists, even with a loss of Sia/Twn inputs. (C) Expression of the *Gsc* promoter-lacZ reporter containing mutations in the homeodomain binding site within the Nodal responsive DE. Expression persists, even with a loss of Nodal inputs. (D) Expression of the *Gsc* promoter-lacZ reporter containing mutations both the PE and DE. Expression is lost without Wnt or Nodal inputs. (E) Uninjected embryos.

regulatory requirements of *Gsc*, as well as to test the importance of the spacing between the DE and PE or the requirement for the identified FoxH1 binding site within the PE (Labbe et al., 1998). Additionally, these reporters may be used to generate transgenic mice, which could help to clarify the importance of the Nodal and Wnt responsive elements in *Gsc* regulation during early mouse development. For instance, while the mouse PE retains Wnt-responsiveness in *Xenopus* ectodermal explants (Watabe et al., 1995), it remains unclear whether Wnt plays a role in *Gsc* expression in the mouse. These constructs may also help in the identification of potential regulators of *Gsc* expression in mammalian embryogenesis.

5.5. Interactions of Wnt and Nodal in Other Contexts

The Wnt and Nodal pathways are important to a number of processes during development and adult life. Recently, combinations of signaling factors have been used to induce the formation of specific tissue types from embryonic stem cells (ES cells). It is thought that ES cells would need to go through the same fate specification process as cells of the embryo. For example, a requirement for BMP, Wnt and Activin signals is important for the specification of blood cells from ES cells. These signals presumably act by inducing formation of the mesodermal lineage with Wnt and Activin, and ventralizing that lineage with BMP signals, as occurs in the embryo (Nostro et al., 2008). Similarly, the specification of insulin producing cells from a population of human ES cells requires inputs from both Wnt and Nodal signaling, presumably by specifying the endodermal lineage through Nodal signaling, and then posteriorizing that tissue with Wnt signaling (Nostro et al., 2011).

While these pathways are indeed important in specifying multiple tissue types

and cell lineages, the interactions of effectors of these pathways in these contexts is not yet well understood. A combination of Smad and Wnt signaling is also involved in the expression of *Twn* during early blastula stages (Labbe et al., 2000; Nishita et al., 2000), suggesting that Wnt and Nodal signals are interacting prior to the onset of organizer gene expression. Both Smad2 and Smad3 form a complex with β-catenin and TCF/LEF, resulting in enhanced transcriptional activation of target genes and changes in cell fate specification (Guo et al., 2008; Shafer and Towler, 2009). With a large number of Wnt and Nodal pathway members, it is likely that members of each signaling pathway can interact with other signaling pathways at multiple levels to produce changes in specification or differentiation of tissues. Elucidation of these mechanisms of interaction will allow a clearer picture of the complex roles signaling pathways play in tissue formation and morphogenesis.

5.6. Integration of Signals During Development

The integration of signals at target promoter regions is important in many aspects of development and adult life. For example, recent work to identify important regulatory domains in cardiac development involved ChIP-seq to identify regions bound by multiple known cardiac transcription factors (He et al., 2011). In the past, enhancer regions were identified by the binding of common co-activators, such as p300 or the SWI/SNF component Brg1 (Rada-Iglesias et al., 2011; Visel et al., 2009). This study identified regions that did not overlap with previously identified enhancer regions (He et al., 2011), suggesting that co-activator occupancy cannot predict the location of all enhancer sequences. Seven of these previously unidentified domains drove expression of a reporter in cardiac tissue in the mouse, suggesting that developmentally relevant

regulatory domains can be identified by the binding of multiple transcription factors (He et al., 2011). To apply this idea to our work, the identification of genomic regions containing conserved homeodomain and Fox binding sites in close proximity may reveal new developmental regulatory domains, and perhaps new factors important in organizer function or formation.

The recent identification of the Yamanaka factors (Oct4, Sox2, Nanog) that induce differentiated cells to become stem cell-like cells (called iPS cells) further emphasizes the importance of transcription factor interactions at gene regulatory domains (Takahashi and Yamanaka, 2006). These iPS cells can contribute to most, if not all, of the tissues of the embryo (Okita et al., 2007), suggesting that these cells have been converted from a differentiated fate to a more plastic, stem cell fate. These transcription factors cooperatively activate pluripotency genes and repress genes resulting in differentiation, suggesting that interactions among these transcription factors are essential to maintaining cells in a stem-cell like state (reviewed in Jopling et al., 2011; Yamanaka and Blau, 2010). While we still understand very little about the process involved in the formation of iPS cells, identification of domains bound by these transcription factors has revealed a large number of genes that contribute to pluripotency. The iPS cell itself is quite intriguing for the therapeutic implications of a de-differentiated cell generated from a patient's own tissue. Elucidation of the ways that transcription factors, such as Oct4 and Sox2, or Sia/Twn and FoxH1, for that matter, integrate signals at target promoters is essential in understanding how gene transcription influences choices in cell fate and differentiation.

5.7. Conclusion

In summary, we find that the Wnt effectors Sia and Twn are required together for the formation of the organizer in *Xenopus laevis*. Sia/Twn activate expression of the organizer gene *Gsc* through a conserved P3 site located within the Wnt responsive PE of the promoter. The Nodal pathway effectors FoxH1 and Smad2/3 cooperate with Sia/Twn in the expression of three organizer genes, *Gsc*, *Cer* and *Chd*. A transcriptional complex, consisting of Nodal and Wnt pathway effectors, along with the common co-activator p300, forms at the promoters of these genes in response to active signaling from both pathways. The formation of this complex at three endogenous promoters suggests that this may be a common regulatory strategy important for the expression of most, if not all, organizer genes. Integration of signals from two pathways at the promoters of multiple organizer genes implies that activation of multiple signaling pathways during development can lead to the formation of uniquely active transcriptional complexes that result in boundaries of gene expression and tissue formation. Our work suggests that a complex of Wnt and Nodal effectors during the early gastrula stage is essential in the expression of organizer gene during organizer formation.

Appendix I Mechanisms of Repression by Groucho Co-Repressors

AI.1 Summary

Concise and controlled gene expression is an essential factor in the development and maintenance of all tissues. While much is known about activation of gene expression, the idea of active repression of target genes is still being widely explored as an essential part of most signaling pathways. This review will focus on the Groucho/Transducin-like Enhancer of Split family of proteins, a common group of co-repressors, and their known and predicted mechanisms of transcriptional repression. Groucho/TLE family members are unable to bind DNA and thus are recruited to target genes by DNA-bound transcription factors, where they recruit co-factors to repress target gene transcription (reviewed in Jennings and Ish-Horowicz, 2008)). A recent paper by Sekiya and Zaret challenges the current model of Groucho dependent transcriptional repression, implying that Gro/TLE family members have intrinsic chromatin remodeling activity in the absence of recruited co-factors (Sekiya and Zaret, 2007). This review will explore the current models for Groucho-dependent repression and will emphasize areas where more work is needed to determine how Gro/TLE family members are acting at target promoters.

AI.2 General Mechanisms of Repression

Repression of target genes occurs in a number of different ways, such as post-translational modifications to histones, ATP dependent chromatin remodeling, and DNA methylation. Transcriptional repressors can also interfere directly with transcriptional activators by blocking access to promoter binding sites or interacting with members of

the Mediator complex or RNA polymerase II to prevent re-initiation of transcription. Current evidence suggests that the Gro/TLE family of proteins can repress transcription through the recruitment of the chromatin modifier histone deacetylase 1 (HDAC1) (Brantjes et al., 2001; Chen et al., 1999) as well as by directly interacting with transcriptional activators and members of the mediator complex (Cai et al., 2003; Zhang and Emmons, 2002). This review will explore the current understanding of the mechanism of Gro/TLE mediated repression of genes through interactions with a number of diverse transcription factors.

AI.3 Nomenclature

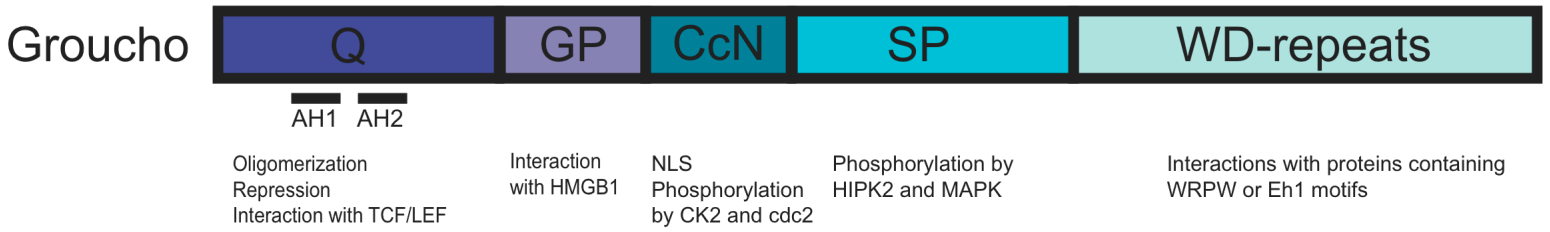
The Groucho allele was initially identified in a screen in *Drosophila melanogaster* (Lindsley, 1968). The mutation identified gave the flies extra bristles above their eyes and was named in homage to the comedian and actor Groucho Marx (Lindsley, 1968). Since the discovery of Groucho, homologs have been identified in other invertebrates as well as vertebrates, including frog, mouse and human. The frog and mouse Groucho genes were originally called Groucho Related Genes (Grg) (Mallo et al., 1993; Molenaar et al., 2000), while the human forms of Groucho were identified as Transducin-Like Enhancer of split (TLE) (Molenaar et al., 2000; Stifani et al., 1992). This review will refer to the Groucho and TLE family of genes as the Gro/TLE family, while reference to particular genes in particular species will be referred to as Gro in *Drosophila*, and Grg/TLE in vertebrates. *Drosophila* has only one Gro gene, while vertebrates have four Gro/TLE genes (termed Grg1-4, TLE1-4, respectively). There are two truncated versions of Grg/TLE in vertebrates, termed Grg5 and AES (Amino-terminal Enhancer of Split) that

consist only of the Grg N terminus. The role of these short Grg/TLE family members will be discussed in further detail in this review.

AI.4 Structure of Groucho

The Gro/TLE family of proteins contains several highly conserved domains including the Q (glutamine rich) domain, the Glycine/proline rich domain (GP), the CcN domain (with CK2 and cdc2 phosphorylation sites and Nuclear localization signal), the serine/proline rich domain (SP) and the WD40 domain, consisting of tryptophan and aspartic acid repeats (reviewed in Buscarlet and Stifani, 2007)) (Fig AI.1). The N-terminal Q domain has shown to be important for oligomerization, transcriptional repression, and protein/protein interactions (reviewed in Buscarlet and Stifani, 2007). Tetramerization of the Gro/TLE family of proteins is thought to facilitate spreading of the repressive signal along the chromatin (Chen et al., 1998; Song et al., 2004). Gro/TLE both homo- and hetero-tetramerize (Chen et al., 1998), and it was believed that this interaction was necessary for Gro/TLE function *in vivo* (Song et al., 2004). However, a recent paper by Jennings *et al.* suggests that Gro tetramerization may not be required for the whole of Gro function *in vivo* (Jennings et al., 2008). The GP domain, C terminal to the Q domain, is important for interactions with Rpd3/HDAC1, a histone deacetylase shown to interact directly with Gro/TLE family members to modulate repressive activity (Chen et al., 1999). The combination of the Q and GP domains are the minimum domains shown to be required for repression when fused to a heterologous DNA binding domain (Fisher et al., 1996). The CcN domain contains regions phosphorylated by CK2 (casein kinase 2) (Nuthall et al., 2004) and cdc2 (cell division cyclase 2) (Nuthall et al., 2002), while the SP domain contains regions phosphorylated by HIPK2 (homeodomain

Figure AI.1 A schematic of the conserved Groucho domains



Q, glutamine rich repeats; AH1, AH2, predicted amphipathic helices; GP, Glycine and proline rich domain; CcN, CK2 and cdc2 phosphorylation sites near nuclear localization signal; SP, serine/proline rich domain; and WD domain which mediates many protein proteins interactions with transcriptional repressors.

interacting protein kinase 2) (Choi et al., 2005) and MAPK (mitogen activated protein kinase) (Cinnamon et al., 2008; Hasson et al., 2005). These phosphorylation sites suggest that Gro/TLE activity can be controlled by post-translational modifications. The WD40 domain (also known as the WDR domain) forms a structure called the β-propeller and is important for interactions with DNA bound transcriptional repressors containing the WRPW or Eh1 (Engrailed homology 1) motifs (Jennings et al., 2006).

AI.5 Homology with Yeast TUP1/SSN6

While a bona-fide Gro/TLE family member in yeast does not exist, there is a similar repressor called Tup1/SSN6 that is important in mediating repression of a number of genes (reviewed in Malave and Dent, 2006)). The domain structure of TUP1 has been shown to be homologous with metazoan Gro/TLE (Flores-Saaib and Courey, 2000). TUP1 exists as a tetramer and contains an N-terminus region which folds into a helical structure important for tetramerization and interactions with SSN6 (Jabet et al., 2000). The C-terminus of TUP1 has 7 WD40 repeats, forming a 7-bladed propeller structure important for protein-protein interactions (Green and Johnson, 2005; Sprague et al., 2000), as seen in Gro/TLE (Jennings et al., 2006). SSN6 has 10 tetratricopeptide repeats (TPRs) that form a superhelical cavity that accommodates the TUP1 N-terminal tetramer (Jabet et al., 2000). It appears that each TPR is required in different repressive scenarios (Tzamarias and Struhl, 1995), suggesting that TUP1-SSN6 are flexible in conformation as well as target gene repression (Malave and Dent, 2006). The TPRs are also important in interactions with different HDACs (Davie et al., 2003; Davie et al., 2002). TUP1/SSN6 can interact with the tails of Histones 3 and 4 (H3, H4, respectively) and this interaction is carried out by two regions in the N terminus of TUP1 (Edmondson

et al., 1996). This association prefers hypoacetylated H3 and H4 (Edmondson et al., 1996), a trait shared with Gro/TLE (Flores-Saaib and Courey, 2000), as will be discussed later. Hyperacetylated histones prevent TUP1/SSN6 mediated repression (Watson et al., 2000). Histones near TUP1/SSN6 recruitment sites *in vivo* are hypoacetylated (Bone and Roth, 2001; Davie et al., 2002).

TUP1/SSN6 have gene specific effects on chromatin, depending on where localized. Repression can spread across chromatin or can be localized to very distinct regions, implying that TUP1/SSN6 may use different repression strategies reliant on transcription factor recruitment, promoter architecture or other factors (Malave and Dent, 2006). Similarly, deletion of different HDACs in yeast abrogates only some TUP1/SSN6 mediated repression (Davie et al., 2003; Davie et al., 2002), again indicating that TUP1/SSN6 have several mechanisms of transcriptional repression. In some instances, TUP1/SSN6 recruitment can lead to changes in nucleosome positioning (Fleming and Pennings, 2001; Ganter et al., 1993; Kastaniotis et al., 2000; Li and Reese, 2001; Saito et al., 2002; Shimizu et al., 1991), indicating that TUP1/SSN6 recruit ATP-dependent chromatin remodelers to specific target genes. TUP1/SSN6 also associate with members of the Mediator complex and RNA polymerase II (Carlson, 1997; Chen et al., 1993; Gromoller and Lehming, 2000; Kuchin and Carlson, 1998; Papamichos-Chronakis et al., 2002; Song and Carlson, 1998), which is suggested to prevent transcription re-initiation or to directly block transcription of target genes (Malave and Dent, 2006). Consistent with the idea that TUP1/SSN6 have different effects on different target genes, diverse histone acetylation patterns are associated with TUP1/SSN6 bound target genes (Deckert and Struhl, 2001; Watson et al., 2000), implying that recruitment of different HDACs leads to different histone acetylation patterns at target genes. Histone

methylation does not seem to play a role in TUP1/SSN6 mediated repression, as deletion of histone methyltransferases (HMTs) in yeast has no effect on TUP1/SSN6 target gene repression (Malave and Dent, 2006). Taken together, TUP1/SSN6 share several homologous functions with Gro/TLE and seem to be extremely flexible co-repressors, recruiting co-factors with varying effects on target gene repression and causing different patterns of chromatin modification in a context-dependent manner. It is yet to be demonstrated whether Gro/TLE act as such flexible co-repressors, but some data suggest that Gro/TLE may have different effects on repression depending on the DNA bound factor recruiting Gro/TLE.

AI.6 Factors that recruit Gro/TLE

The Gro/TLE family of proteins have been shown to interact with many different transcription factors in many different types of tissues, including ones that are known to function as both transcriptional activators and repressors. For example, Gro/TLE have been shown to interact with basic helix-loop-helix proteins such as Hairy and Hes, Runt homology domain proteins such as AML, Fox family proteins, homeodomain proteins, Tcf/Lef related HMG box proteins and many others (reviewed in Buscarlet and Stifani, 2007)). The main way that Gro/TLE interact with these factors is through either a WRPW motif or an Eh1 motif (reviewed in Buscarlet and Stifani, 2007)). For example, the Forkhead box (Fox) transcription factor family member FoxD3 functions as a transcriptional repressor during the formation of the mesodermal germ layer in early development (Yaklichkin et al., 2007). FoxD3 contains an Eh1 motif which is essential for Grg4 interaction and for FoxD3 mediated transcriptional repression (Yaklichkin et al., 2007). In *Drosophila*, the transcription factor Dorsal acts as both a repressor and an

activator and is essential for dorsal/ventral patterning of the developing embryo (Ratnaparkhi et al., 2006). Gro interacts with Dorsal by a modified WRPW domain that allows for weakened recruitment of Gro. Therefore, Dorsal dependent recruitment of Gro depends on other factors to aid in Gro recruitment, allowing for spatial control of Dorsal dependent repression (Ratnaparkhi et al., 2006).

AI.7 Role of Gro/TLE in Development

Gro/TLE has been shown to play an important role in a number of developmental contexts. Gro was first shown to interact with the Hairy and Hairy related group of the basic-helix-loop-helix (bHLH) protein family (Paroush et al., 1994). The interaction of Gro with these bHLH proteins is essential for segmentation, sex determination and neurogenesis downstream of the Notch signaling pathway in the developing fly (Paroush et al., 1994). The WRPW motif at the C-terminus of these bHLH proteins is essential for the interaction with Gro. Interestingly, the original paper shows that the WD40 domain is not essential for the interaction with Hairy *in vivo* (Paroush et al., 1994), but a later paper identifies the WD40 domain as facilitating the interaction with the WRPW motif of Hairy (Jennings et al., 2006). While the crystal structure of the WD40 domain identifies the contacts the WRPW domain makes with the WD40 β-propeller, it is not clear whether other regions of Gro/TLE may facilitate this interaction or whether other regions of Gro/TLE interact with WRPW proteins under different circumstances.

In *Xenopus laevis*, Grgs have been shown to be important in anterior/posterior patterning as well as germ layer formation. Grgs interact with Tcf-3, an effector of the Wnt signaling pathway that acts as both a repressor and an activator (Roose et al., 1998). It is in its repressive form when bound by Grg and it is activated when bound by

β-catenin (Brantjes et al., 2001; Roose et al., 1998). In sea urchin, LvGroucho was found to repress β-catenin/Tcf signaling (Range et al., 2005). LvGroucho was shown to interact with Tcf through the Q and WD domains and to functionally compete with β-catenin for binding to Tcf (Range et al., 2005). In zebrafish, Grgs has been shown to interact with several different transcriptional repressors to modulate shield formation, somitogenesis, segmentation of the hindbrain, and central nervous system development, including the eye and the forebrain (Kobayashi et al., 2001; Nakada et al., 2006; Runko and Sagerstrom, 2003; Shimizu et al., 2002). In chick and mouse, Grgs are thought to play a role in hematopoiesis, neurogenesis and somitogenesis (Javed et al., 2000; Van Hateren et al., 2005; Yamagata et al., 2005). With many Grg/TLE proteins and many roles in development, it may be difficult to determine the roles of individual Grg/TLE proteins. Limited expression data suggests Grg/TLEs play conserved roles in mammalian development (Yao et al., 1998). Grg/TLEs show overlapping and distinct expression patterns in many tissues in mouse and human (Dehni et al., 1995). Because Grg/TLE is thought to tetramerize, it is interesting to speculate what roles Grg/TLE hetero-tetramers play during development of specific tissues. Similarly, it is unclear how much redundancy exists within the vertebrate Grg/TLE family.

AI.8 Methods of Repression

The current model for Gro/TLE-mediated transcriptional repression involves the recruitment of Gro/TLE to target promoters by a DNA bound transcription factor (reviewed in Gasperowicz and Otto, 2005; Jennings and Ish-Horowicz, 2008)). Gro/TLE recruits histone modifiers, such as HDAC1, to de-acetylate histone tails, thus allowing chromatin to condense to repress transcription [1,60]. Gro/TLE have been shown to

interact with members of the Mediator complex, which suggest that Gro/TLE is directly inhibiting Mediator interactions with promoter regions (Zhang and Emmons, 2002). Because of the ability of Gro/TLE to tetramerize, it is thought that once established, the Gro/TLE repressive state can then spread along the chromatin, leading to a stable repressive state (Gasperowicz and Otto, 2005; Jennings and Ish-Horowicz, 2008).

The general idea of repression is that once DNA is compacted into a denser chromatin structure, transcriptional activators cannot access particular promoter or enhancer regions, thus preventing transcriptional activation. Compact chromatin structure can be established in a number of ways: histone modifications, methylation of the DNA itself, as well as nucleosome positioning and localization within the nuclear compartments. Histone modifications are carried out by the recruitment of histone modifiers, such as HDACs and histone methyltransferases (HMTs). Gro/TLE interact with the histone tails of both Histone H3 and Histone H4 (Flores-Saaib and Courey, 2000; Palaparti et al., 1997). DNA methylation plays a large role in inherited and broad range silencing, but its role in Gro/TLE mediated repression has not yet been determined. Nucleosome positioning can lead to impeding or physically blocking binding by transcriptional activators. ATP-dependent chromatin remodelers, such as SWI/SNF complexes or the BRG complex, can carry out movement or replacement of nucleosomes. At this time, there is no evidence that ATP-dependent remodelers are recruited by the Gro/TLE co-repressors, but evidence does suggest TUP1/SSN6 recruit ATP-dependent chromatin modifiers (reviewed in Malave and Dent, 2006)). Published reports indicate that certain phosphorylated versions of Gro/TLE are more closely associated with the nucleus than others (Husain et al., 1996). However, it is not yet clear what the significance of this association is with regards to transcriptional repression or

the effect on association with DNA bound factors.

AI.9 Gro/TLE Interaction with Histones

Because Gro/TLE share several structural similarities with TUP1, investigations were carried out to determine if the Gro/TLE repressive mechanism is similar to that of TUP1. TUP1 interacts with nucleosomes and this interaction is required for TUP1-mediated repression (Edmondson et al., 1996). Both Gro and human TLE were shown to associate with histones, particularly histone H3 (Flores-Saaib and Courey, 2000; Palaparti et al., 1997). In the case of Gro, the N-terminal Q domain is sufficient and necessary for this interaction *in vitro* (Flores-Saaib and Courey, 2000; Palaparti et al., 1997). Gro constructs containing several mutated residues that abrogate histone binding also display reduced repressive ability in S2 cells (Flores-Saaib and Courey, 2000). Gro also preferentially binds hypoacetylated histone tails *in vitro* (Flores-Saaib and Courey, 2000), suggesting that Gro maybe unable to establish a repressive state if chromatin contains acetylated histone tails. This raises the question of how Gro/TLE might establish repression, if these co-repressors modulate repression through de-acetylation. However, these findings have not been substantiated *in vivo*. In the recently published paper by Sekiya and Zaret, Grg3 binds chromatin, requiring the C-terminal WD40 domain to create compacted chromatin (Sekiya and Zaret, 2007), contradicting the previous findings that the N-terminal Q domain is necessary and sufficient for histone binding. Binding of Grg3 alone to chromatin *in vitro* created a visibly denser chromatin structure (Sekiya and Zaret, 2007). At least two nucleosomes were required for this structure to form, suggesting that Grg3 creates a more compact chromatin structure by condensing nucleosomes together and spreading along the chromatin (Sekiya and

Zaret, 2007). This chromatin structure is DNaseI sensitive, and protease analysis of the Grg3-chromatin complex indicates that Grg3 undergoes a conformational change upon binding to chromatin (Sekiya and Zaret, 2007). This is the first demonstration that Grg3 alone has intrinsic chromatin remodeling capacity, without associated HDACs or other co-factors, and likely suggests that Gro/TLE based repression acts through multiple mechanisms in a context dependent manner.

AI.10 Gro/TLE Interaction with Chromatin Modifying Enzymes

Gro has been shown to interact with Rpd3 (HDAC1) genetically, biochemically and at the level of transcription (Chen et al., 1999; Choi et al., 1999). The GP region of Gro is required for this interaction and inhibition of HDAC activity abrogates Gro-dependent repression (Chen et al., 1999). In early Drosophila embryogenesis, Rpd3 and Gro are expressed in similar places and embryos deficient for both Gro and Rpd3 display a more severe pair-rule type phenotype that either deficiency alone (Chen et al., 1999). However, Rpd3 mutants do not share many characteristics that Gro mutants have, specifically the neurogenic phenotype (Chen et al., 1999; Jennings and Ish-Horowicz, 2008), suggesting that Gro acts either through more than one HDAC for repression, or that Gro can use several different strategies for repression, as seen in TUP1/SSN6 mediated repression. Subsequently, all long forms of Grgs were shown to interact with HDAC1 (Brantjes et al., 2001), implying this is a mechanism of repression shared by the whole Grg/TLE family. The short forms of Grg/TLE were unable to interact with HDAC1, suggesting that these forms might act as naturally occurring dominant negative forms of Grg/TLE (Brantjes et al., 2001).

AI.11 Grg/TLE Interactions with Transcriptional Effectors

The *C. elegans* homolog of Gro, called Unc-37 genetically interacts with components of the Mediator complex (Zhang and Emmons, 2002). Drawing again from work with TUP1, a genetic interaction was identified between Unc-37 and Mediator, in which mutation of Unc-37 along with mutation in components of mediator led to a more severe loss of male sensory neurons (Zhang and Emmons, 2002). Although not surprising that a repressor acts by physically interacting with mediators or effectors of transcription, this interaction has yet to be explored in other organisms and the mechanism of the interaction and their effects on transcriptional repression has not yet been defined.

During embryonic development, the Pax2 transcription factors are important in regulating kidney and nervous system development and can act as both transcriptional activators as well as repressors. Pax2 is phosphorylated by active c-Jun N-terminal kinase (JNK) to enhance transcriptional activation (Cai et al., 2003). Grg4 inhibits this phosphorylation event, preventing Pax2 transcriptional activation (Cai et al., 2003). Interaction with Grg4 depends on Pax2 DNA binding and does not require histone deacetylation (Cai et al., 2003). This study demonstrates that Gro/TLE can manipulate transcriptional repression by directly interacting with DNA bound transcription factors, without HDAC activity (Cai et al., 2003). These results imply that Gro/TLE act through different mechanisms dependent on the DNA-bound transcription factors recruiting Gro/TLE or the genomic structure of target genes.

AI.12 Gro/TLE Conformation

In S2 cells, Gro forms a tetramer and that the Q domain is required for this tetramerization (Chen et al., 1998). Mutation of important residues contributing to the leucine zipper structure within the Q domain reduced Gro tetramerization ability as well as its repressive abilities, both *in vitro* and *in vivo*, indicating that the leucine zipper structure is essential for tetramer formation (Chen et al., 1998; Song et al., 2004). When Gro was tethered to the Gal4 DNA binding domain (Gal4DBD), it was able to repress target genes, regardless of the position of where the Gal4DBD bound, suggesting that Gro repression could spread along the DNA (Chen et al., 1998). This repressive ability was also lost when the leucine zipper was mutated, implying that tetramerization is required for the spreading of Gro dependent repression (Chen et al., 1998). Replacing the Q domain of Gro with the defined tetramerization domain of the transcription factor p53 allowed repression, suggesting that the Gro Q domain solely functions to tetramerize (Chen et al., 1998). Additionally, overexpression of Gro in the Drosophila wing disk causes developmental defects, while overexpression of the mutant Gro which cannot oligomerize has no effect, further indicating that Gro activity depends on tetramerization (Song et al., 2004).

However, a recent paper published suggests that Gro activity is not always dependent on tetramerization (Jennings et al., 2008). Gro alleles that have mutations in the Q domain region were isolated, and their phenotype is less severe than that of a full Gro null phenotype (Jennings et al., 2008). These alleles were shown to encode Gro protein that is unable to tetramerize *in vitro*, yet flies exhibited a relatively mild phenotype, indicating either that Gro can tetramerize with another previously unidentified method or Gro tetramerization is not required for all aspects of Gro function (Jennings et

al., 2008). Similarly, another recent study found that the Grg3 WD40 domain is necessary for condensation of chromatin but that the Q domain has a lesser contribution to condensing chromatin at FoxA1 target genes *in vitro* (Sekiya and Zaret, 2007). These studies indicate that the previous model for Gro/TLE in which Gro/TLE formed tetramers allowing for spreading of repression along the chromatin might not be the only mechanism through which Gro/TLE function. It is now clear that Gro acts through more than one method of transcriptional repression, and that the *in vivo* function of Gro oligomerization will need further characterization on a case-by-case basis.

The WD40 domain forms a β-propeller structure to which factors containing either the WRPW domain or the Eh1 motif can bind (Jennings et al., 2006). The WRPW motif is compacted into this region, while the Eh1 motif creates a helical structure, indicating why the Eh1 motif can vary so much in its amino acid content, but the WRPW motif has very few variations, and those variations mostly lead to weaker association with Gro (Jennings et al., 2006). An early Gro paper found that the WD40 domain of Gro is not essential for interactions with the WRPW domains of hairy and other related bHLH proteins in a yeast two hybrid interaction assay, and it was thought that the SP domain could mediate some of the Gro/bHLH protein interaction, although this finding has yet to be substantiated *in vivo* (Paroush et al., 1994).

AI.13 Gro/TLE Modulation by Post-Translational Modifications

In researching Gro/TLE modifications, many groups have shown that Gro/TLE is phosphorylated *in vivo* under many different circumstances (Cai et al., 2003; Choi et al., 2005; Husain et al., 1996; Nuthall et al., 2002; Nuthall et al., 2004). Gro/TLE does contain several putative phosphorylation motifs within the CcN and SP domain. Drug

stimulation of CDC2 (CDK1) increases the phosphorylation state of Gro/TLE in cell culture (Nuthall et al., 2002). Phosphorylation maps to the CcN domain of Gro/TLE and the hyperphosphorylated Gro/TLE is seen at the G2/M cell cycle transition and correlates with a reduced association with the nucleus, as shown in fractionation studies (Nuthall et al., 2002). When CDC2 activity is inhibited, Gro/TLE-dependent repression is increased, implying that phosphorylation can negatively regulate Gro/TLE activity (Nuthall et al., 2002). Reduced association with the nucleus suggests that Gro/TLE dissociate from DNA in a cell-cycle dependent manner, but it is not yet clear what role this phosphorylation event would play *in vivo* (Nuthall et al., 2002).

Gro/TLE is phosphorylated by CK2 in the CcN domain (Nuthall et al., 2004). Gro/TLE contain a conserved serine at position 239 which, when mutated, reduces hyperphosphorylation of Gro/TLE and reduces nuclear association and repression (Nuthall et al., 2004). Phosphorylation at S239 increases Gro/TLE1 association with Hes-1, a Notch pathway effector, and increases Gro/TLE-dependent repression (Nuthall et al., 2004). This phosphorylation event is shown to be important during neuronal differentiation, when Hes-1 dependent repression through Gro/TLE1 is required (Nuthall et al., 2004). In order to determine the mechanism of Hes-1 dependent activation, the Hes-1/Gro/TLE repressive complex was isolated (Ju et al., 2004). The poly (ADP-ribose) polymerase1 (PARP-1) was isolated from the Hes-1 repressive complex and acts as a molecular “switch” that turns Hes-1 from a repressor into an activator (Ju et al., 2004). Activation of PARP-1 by Ca^{++}/Calmodulin dependent kinase II (CaMKIIδ) in response to a calcium signal leads to poly-ADP ribosylation of Grg1/TLE1 and associated factors, causing dissociation from Hes-1 to relieve repression (Ju et al., 2004).

In another example of the relief of repression, Gro is phosphorylated by dHIPK2

(Choi et al., 2005). Phosphorylation of Gro at Ser297 promotes dissociation of Gro from DNA bound factor eyeless (Pax6) and HDAC1, leading to relief of repression (Choi et al., 2005). The authors suggest a model in which some signal "X" activates dHIPK2, leading to phosphorylation of Gro and reduced repression (Choi et al., 2005).

Gro/TLE is also phosphorylated in response to activated receptor tyrosine kinase (RTK) activity (Cinnamon et al., 2008; Hasson et al., 2005). MAPK phosphorylates Gro at Thr308 in the SP domain and Ser510 in the WD40 domain in response to EGF pathway activation (Hasson et al., 2005). This modification downregulates Gro activity, and diminishes Hes based repression, indicating a new way in which the EGF pathway interacts with the Notch pathway (Hasson et al., 2005). Gro is also phosphorylated in response to other RTK pathways, such as Torso and FGF in the developing *Drosophila* embryo (Cinnamon et al., 2008). The persistence of Gro phosphorylation long after the RTK signal is gone implies that phosphorylation of Gro may lead to long term inhibition of Gro activity (Cinnamon et al., 2008). Although phosphorylated Gro still associates with the nucleus and DNA bound partners hairy and odd-skipped as well as Rpd3, the authors speculate that Gro can no longer form functional complexes when phosphorylated (Cinnamon et al., 2008).

AI.14 Establishment or Maintenance of Repression

An elegant paper from the Gergen lab set out to discern between the establishment and the maintenance of a repressive signal by looking at Runt-dependent repression of engrailed (en) during segmentation of the Drosophila embryo (Wheeler et al., 2002). In a modified suppressor screen, three co-repressors of Runt were identified: Gro, c-terminal binding protein (CtBP), Rpd3 and tramtrack (ttk) (Wheeler et al., 2002).

When looking at the interaction of these factors with Runt, a timing difference was noticed in the rescue of Runt-dependent repression of en (Wheeler et al., 2002). Runt interacts with Gro through a VWRPY motif, a modified version of the WRPW motif (Wheeler et al., 2002). Deletion of this motif in Runt can still repress en expression, but only for a certain time (Wheeler et al., 2002). The typical stripes of en expression is re-established at a later time, which was unexpected (Wheeler et al., 2002). The authors conclude that the Runt-Gro interaction is not required for establishment of en repression at the early blastoderm state, but is required for maintenance of this repression (Wheeler et al., 2002). If ttk expression is reduced, the proper pattern of en repression is not established, but one might hypothesize that the proper pattern of en repression would be established later on by Runt-Gro interactions (Wheeler et al., 2002). However, this is not the case; en repression is not re-established, indicating that ttk dependent establishment of en repression is necessary for Gro-dependent maintenance of en repression (Wheeler et al., 2002). The authors suggest a two-step model for Runt dependent repression that requires ttk for establishment and Gro/Rpd3 for maintenance (Wheeler et al., 2002). These findings indicate a previously unappreciated idea in Gro/TLE dependent repression: that Gro/TLE may be required in maintaining a repressive state, but not in establishing that state. Previous findings that Gro/TLE associates preferentially with hypoacetylated histones (Flores-Saaib and Courey, 2000) supports this idea, but further work is needed in order to discern how Gro/TLE may function in repressive establishment or maintenance.

AI.15 The short Gro/TLE forms

A number of short Gro/TLE proteins have been identified, including Grg5 in

mouse and frog (Brantjes et al., 2001; Mallo et al., 1993), AES, (Amino-terminal Enhancer of Split) the Grg5 homolog in humans (Miyasaka et al., 1993), as well as alternative splice forms of Grg1 (Grg1S) (Lepourcelet and Shivdasani, 2002), Grg3 (Grg3B) (Leon and Lobe, 1997) and TLE4 (QD) (Milili et al., 2002). All of these short forms of Gro/TLE consist of only the Q and GP domains and, as such, are thought to act as dominant negative forms of Gro/TLE that relieve repression of Gro/TLE target genes (Brantjes et al., 2001; Gasperowicz and Otto, 2005). However, several lines of evidence exist that show that these short forms of Gro/TLE act as both repressors and activators, implying that these short forms of Gro/TLE function separately from the Gro/TLE long forms. Grg5 is the only Gro/TLE gene to be knocked out in mouse (Mallo et al., 1995). Targeted disruption of Grg5 leads to delayed or absent growth of pups and death within five weeks of birth (Mallo et al., 1995). Others exhibit a much slower growth rate and ultimately can survive, but are much smaller than wild type siblings (Mallo et al., 1995). This growth defect is due to the impaired growth of the long bone growth plates and decreased amount of trabecular bone (Wang et al., 2002). However, whether the early lethality phenotype is due to bone outgrowth is not clear. Without examples of knockout of other Gro/TLE family members, it is difficult to determine the mechanism by which Grg5 is functioning.

Grg5 consists of the Q and GP regions of Gro/TLE, but has a very divergent sequence, implying that interactions Gro/TLE can make with the Q and GP domains may not occur in Grg5 (Mallo et al., 1993; Miyasaka et al., 1993). Indeed, in *Xenopus*, XGrg5 is unable to interact with HDAC1, while the isolated Q and GP regions of XGrg4 interact with HDAC1 (Brantjes et al., 2001). Similarly, human AES is unable to interact with HDAC1 or HDAC3 (Yu et al., 2001). Since interactions with HDAC1 seem to be

important to Gro/TLE function (Chen et al., 1999; Choi et al., 1999), it was assumed the Grg5 could moderate repression. However, Grg5 lacks the WD40 domain (reviewed in Gasperowicz and Otto, 2005)), which makes contacts with the DNA bound factors containing the WRPW or Eh1 motifs (Jennings et al., 2006), suggesting that Grg5 either cannot bind to DNA bound factors or that it functions in a different manner from other Gro/TLE family members. Whether the sequence variations in the GP domains of Grg5 and AES play a role in preventing HDAC1 interactions in not known. Perhaps this sequence variation allows recruitment of other HDAC enzymes, or other chromatin remodeling factors. Or, perhaps HDAC recruitment is not the only way Gro/TLE repress transcription.

AES acts as a co-repressor with p65, a member of the NF-κB signaling pathway (Tetsuka et al., 2000). AES expression represses p65 dependent gene transcription, even when stimulated by TNF-α (Tetsuka et al., 2000). Similarly, androgen receptor (AR) interacts with AES to repress ligand dependent transcription in a cell-free system (Yu et al., 2001). AES interacts with TFIIE, a member of the basal transcription complex, although the *in vivo* significance of this interaction has not yet been determined (Cai et al., 2003; Yu et al., 2001). However, HDAC inhibition with TSA does not affect AES dependent repression (Yu et al., 2001), suggesting that the mechanism AES uses to repress AR target genes does not involve recruitment of HDACs. Similarly, when AES is fused to a heterologous DNA binding domain, it acts as a repressor of Gal4-dependent transcription (Ren et al., 1999).

Grg5 has been shown to inhibit Gro/TLE based repression of many transcription factors, including FoxD3 (Yaklichkin et al., 2007), Hnf-3β (Wang et al., 2000), Runx2 (Wang et al., 2004) and TCF (Brantjes et al., 2001). Since all of these factors use

different motifs for interacting with Gro and are from different classes of transcription factor families, it is difficult to draw conclusions about the mechanism of Grg5 action. In contrast, Grg5/AES do not inhibit repression of Gro/TLE dependent repression of Pax5 (Eberhard et al., 2000), FoxG1b (Ren et al., 1999), and Hes-1 (McLarren et al., 2000). Expression of a splice variant of Grg1, (Grg1S) which contains only the Q and GP domains of Grg1, can repress β-catenin/TCF dependent transcription (Lepourcelet and Shivdasani, 2002). In sea urchin, expression of the AES197, the Q and GP domains of LvGroucho, acts as full length LvGroucho during development (Range et al., 2005). This construct lacks the Rpd3 interaction domain, implying again that Grg5/AES based repression does not require HDAC1 interactions (Range et al., 2005).

Taken together these results suggest several roles for short Gro/TLE forms during development. It appears that Grg5/AES act as both repressors and activators, depending on the context in which they are used. Until the mechanism of repressive action of the Gro/TLE family is known, it may be difficult to elucidate the action of Grg5/AES unless in a context dependent manner.

AI.16 Gro/TLE Function as Chromatin Remodelers

In the recent paper by Sekiya and Zaret, the mechanism of Gro/TLE action on chromatin is explored (Sekiya and Zaret, 2007). In the first paper of its kind to look at the mechanism of Grg3 action on the chromatin of a target gene promoter, the authors find the Grg3 has intrinsic chromatin remodeling capabilities, even in the absence of a DNA bound factor (Sekiya and Zaret, 2007). A model for their findings is presented in Figure AI.2 Grg3 binds chromatin, and specifically requires a di-nucleosome subunit for this chromatin remodeling (Sekiya and Zaret, 2007). The chromatin condenses in response

to Grg3 binding and Grg3 undergoes a conformational change (Sekiya and Zaret, 2007). The DNA is still accessible to DNaseI, but the chromatin is more condensed and can interact with other chromatin, creating aggregates of a high molecular weight (Sekiya and Zaret, 2007). Interestingly, the WD40 domain was most important for this activity (Sekiya and Zaret, 2007), which calls into question the current model of Gro/TLE action. Gro/TLE is thought to be recruited by a DNA bound factor, and once bound recruits HDACs to repress target genes. It is also thought that Gro/TLE forms homotetramers with its N-terminal Q domain to allow for the spreading of the repressive signal along the DNA (reviewed in Jennings and Ish-Horowicz, 2008)). However, Sekiya and Zaret found that the C-terminal WD40 domain is important in chromatin condensing, and the N-terminal region, while displaying slightly reduced chromatin modifying abilities, can still condense chromatin, implying that tetramerization is not necessary for the chromatin modifying abilities of Grg3 (Sekiya and Zaret, 2007). Once Grg3 is recruited to the chromatin by DNA bound factors FoxA1 or Hes1, the chromatin condenses further to form closed, DNaseI resistant chromatin (Sekiya and Zaret, 2007). Expression of Grg3 represses FoxA1 target genes in both mouse and human cells (Sekiya and Zaret, 2007). Looking at Grg3 binding to a target promoter *in vivo*, the authors observe that FoxA1 is bound to its binding site, yet nowhere else, as expected (Sekiya and Zaret, 2007). However, Grg3 binding spreads along the chromatin three to four nucleosomes away (the size of a typical regulatory region) from the FoxA1 binding site, implying that Grg3 is interacting with the chromatin without a DNA bound factor (Sekiya and Zaret, 2007). The FoxA1/Grg3 complex prevents recruitment of RNA PolII and TATA Binding Protein, implying that Grg3 is physically prevents binding of crucial transcriptional activators to a promoter or enhancer region (Sekiya and Zaret, 2007).

Figure AI.1 A model for the method of repression by Grg3

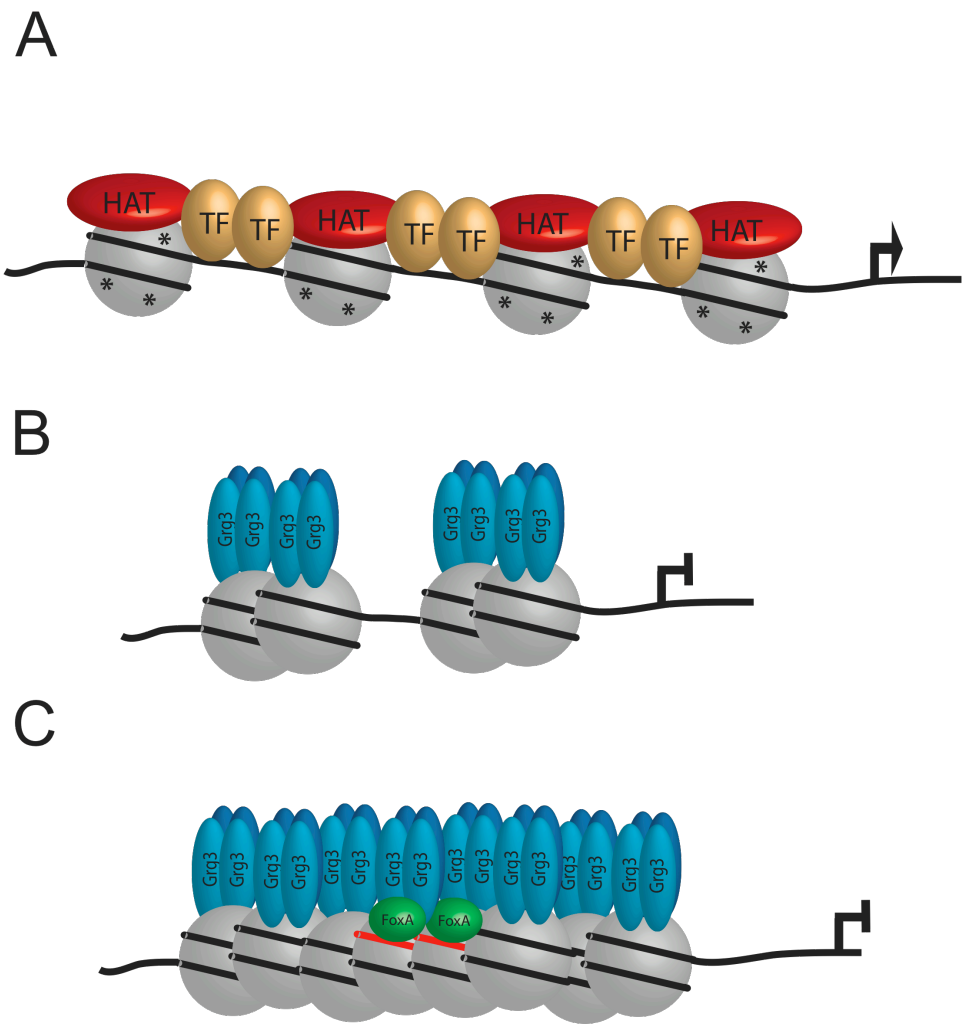


Figure AI.2: **A model for the method of repression by Grg3**.

A) Active transcriptional state with acetylated histones (denoted by *), transcriptional activators (TF) bound to open chromatin and histone acetyltransferase (HAT) recruited to open chromatin and promote transcription. B) Grg3 binds di-nucleosome subunits and condenses chromatin to prevent transcription. C) Upon recruitment of FoxA to its binding site on the DNA (indicated in red), Grg3 recruitment is increased to the site of Fox A recruitment, and also spreads along the chromatin, further condensing chromatin and preventing transcription.

These data raise several questions about the mechanism of Gro/TLE repression. Is DNA binding necessary for transcriptional repression? Are interactions with histone tails sufficient for repression of Grg3 target genes? How is the Grg3 repressive signal spreading along the chromatin; is this due to tetramerization? What role, if any are HDACs playing in this version of Grg3-based repression? Similarly, these data call to mind the different mechanisms TUP1/SSN6 use to repress genes at different promoters. What role does promoter architecture and Gro-DNA bound factor interaction play in the conformation of Gro/TLE co-repressors? Do several conformations of Gro/TLE exist, and does each conformation have a specific repressive mechanism? These and many other questions will hopefully be addressed in the future as more is becoming known about chromatin modifications and transcriptional repression.

AI.17 Conclusions

Throughout this review, it has become clear that Gro/TLE function needs to be evaluated on a case-by-case basis. The publication of the recent paper by Sekiya and Zaret has made an advancement to the study of Gro/TLE transcriptional repressors, but it is clear more work needs to be done. For example, what is the role of Gro/TLE tetramerization? It appears this interaction is not always necessary for Gro/TLE function, since the Drosophila Gro mutants unable to tetramerize present a much milder phenotype than Gro null mutants (Jennings et al., 2008). As such, how does Gro/TLE based repression spread along the chromatin? Does Gro/TLE interact with itself in a number of different ways, or do interactions with different DNA bound factors cause conformational changes in Gro/TLE itself that allows Gro/TLE to behave differently at distinct promoters? In Sekiya and Zaret, the authors find that Grg3 binds chromatin

changing the chromatin conformation, implying Gro/TLE family members have intrinsic chromatin modifying abilities (Sekiya and Zaret, 2007). Their *in vitro* data suggests that transcription factor recruitment is not necessary for this action, implying that Gro/TLE interacts with chromatin without a DNA bound factor (Sekiya and Zaret, 2007). The paper also suggests that Grg3 binding to chromatin spreads along the chromatin, as far as 3-4 nucleosomes away, indicating that Grg3 does not need a DNA bound factor to interact with the chromatin (Sekiya and Zaret, 2007). It will be interesting to see how far along DNA the chromatin structure is affected by Gro/TLE binding, or if, as in the case of TUP1/SSN6, it will be promoter dependent (reviewed in Malave and Dent, 2006)). For example, what histone modifications are seen in the areas of Gro/TLE recruitment? Is there a pattern or are all Gro/TLE target genes different? Could the pattern of histone modification give a clue as to how Gro/TLE or its short counterparts behave at a certain promoter? What role does Gro/TLE play in the establishment versus the maintenance of a repressive signal? Since Gro/TLE have been observed to interact preferentially with hypoacetylated histones (Flores-Saaib and Courey, 2000), are HDACs required before Gro/TLE bind to repress target genes? Are HDACs, in fact, recruiting Gro/TLE family members? Similarly, how does Gro/TLE function in the maintenance of a stable repressive signal versus those temporary repressive signals, as seen during the rapid changes occurring in embryonic development? And lastly, the role of Gro/TLE in vertebrate development is not yet understood. The lack of Gro/TLE knockout animals to understand redundancy and their specific roles in development is required. Altogether, much work needs to be done to understand not only the mechanisms behind Gro/TLE dependent transcriptional repression, but also to understand how gene expression is tightly controlled during development and adult life.

Embryo manipulation and microinjection

Xenopus embryos were collected, fertilized, injected and cultured as previously described (Yao and Kessler, 2001). Embryonic stage was determined according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Ectopic axis induction was scored at the neurula stage as partial axis induction (containing trunk but no head structures) or complete (containing trunk and head structures). Results represent at least five independent experiments. Explants were prepared using a Gastromaster microsurgery instrument (Xenotek Engineering). Capped, *in vitro* transcribed mRNA for microinjection was synthesized from linearized DNA templates using the SP6 mMessage Machine kit (Ambion); 10nl of RNA solution was injected per embryo. Templates for *in vitro* transcription were pCS2+Siamois (Kessler, 1997), pCS2+myc-Siamois (this study, pCS2+myc-SiaQ191E (this study and Kessler, 1997), pCS2+GST-Sia (this study), pCS2+Twn (this study), pSP64-Twin (Laurent et al., 1997), pCS2+myc-Twin (this study), pCS2+GST-Twn (this study), pCS2+myc-FoxH1 (Fast1) (Yaklichkin et al., 2007), pCS2+Xnr1 (Sampath et al., 1997) and pCS2+XWnt8 (Kessler, 1997).pCS2+myc-SiaΔ75 (this study), pCS2+myc-SiaΔ40-75 (this study), pCS2+ myc-SiaΔ39 (this study), pCS2+myc-TwnΔ70 (this study), pCS2+myc-TwnΔ36-70 (this study), pCS2+ myc-TwnΔ35 (this study), pCS2+myc-SiaS12Y (this study), pCS2+myc-SiaΔ40-75S12Y (this study), pCS2+ myc-TwnY12S (this study), pCS2+myc-TwnΔ36-70 Y12S (this study), pCS2+myc-SiaK114A (this study), pCS2+myc-SiaKK126AA (this study), pCS2+myc-SiaK114A,KK126AA (this study), pCS2+TwnK108A (this study), pCS2+TwnKK120AA (this study) and pCS2+TwnK108A,KK10AA (this study).

Plasmid constructs

pCS2+myc-Sia and pCS2+myc-SiaQ191E were generated by PCR amplification of the coding region of Sia or SiaQ191E (Kessler, 1997). The amplified products were subcloned into the BamHI site of pCS2+myc. For pCS2+Twn and pCS2+myc-Twn, the coding region of Twn

(Laurent et al., 1997) was amplified from pSP64-Twn and cloned into the EcoRI site of pCS2+ or pCS2+myc. pCS2+GST-Sia and pCS2+GST-Twn were generated by subcloning the coding regions of Sia or Twn into the XbaI site of pCS2+GST (Yaklichkin et al., 2007). pCS2+myc-SiaΔ75, pCS2+ myc-SiaΔ39, pCS2+myc-TwnΔ70, pCS2+ myc-TwnΔ35 were generated by PCR amplification of Sia or Twn lacking the designated amino acids The amplified products were subcloned into the BamHI site of pCS2+myc for pCS2+ myc-SiaΔ39, the EcoRI site of pCS2+myc for pCS2+myc-SiaΔ75, pCS2+myc-TwnΔ70, and pCS2+ myc-TwnΔ35. pCS2+myc-SiaΔ40-75 and pCS2+myc-TwnΔ35-70 were created using outward directed PCR with pCS2+myc-Sia or pCS2+myc-Twn serving as template. pCS2+myc-SiaS12Y, pCS2+myc-SiaΔ40-75S12Y, pCS2+ myc-TwnY12S, pCS2+myc-TwnΔ36-70 Y12S, pCS2+myc-SiaK114A, pCS2+myc-SiaKK126AA, pCS2+myc-SiaK114A,KK126AA, pCS2+TwnK108A, pCS2+TwnKK120AA and pCS2+TwnK108A,KK10AA were generating using PCR mediated mutagenesis. All constructs were verified by sequencing and *in vitro* translation assays.

For DNAse footprinting, a plasmid containing the –226*Gsc* promoter (Watabe et al., 1995) was digested with BamHI and HindIII and subcloned into pBSII-KS+ to make pBS-226Gsc. pBS-226Gsc was digested with BamHI and HincII for bottom strand labeling, and HindIII and SacII for top strand labeling. For preparation of tagged recombinant proteins, 6xHis- or GST-tagged Sia and Twn were amplified by PCR and subcloned into the pet28b or pGEX vectors, respectively. Reporter constructs with mutations in the *Gsc* promoter sequence were generated by PCR-mediated mutagenesis. Specific mutations introduced into the *Gsc* promoter are indicated in Fig. 2.2A. The *Gsc* luciferase reporter was previously described (Watabe et al., 1995) and was a generous gift of Ken Cho.

Protein purification, pulldown and crosslinking

Histidine-tagged and GST-tagged proteins were purified using standard methods (Novagen and Pharmacia Biotech). The *in vitro* GST pulldown assay was performed as previously described (Yaklichkin et al., 2007). GST or GST-Siamois (2μg) were incubated with full

length His-Sia or His-Twn (2µg), protein complexes were recovered using Glutathione Sepharose 4B (GE Healthcare, 17-0756-01) and subjected to western analysis using an anti-6X His tag antibody (AbCam). For the protein crosslinking studies, EGS (Ethylene Glycol-bis (succinic acid N-hydroxysuccinimide ester) (Sigma, E3257) dissolved in DMSO was added to each protein sample and incubated for 30 minutes at room temperature. DMSO alone was used for control reactions. The crosslinking reaction was stopped by addition of glycine to a final concentration of 75mM. Crosslinking of proteins in the presence of the DNA-binding site was performed in a similar manner by incubating oligonucleotides with proteins for 20 minutes on ice prior to addition of EGS. Crosslinked protein complexes were detected by western analysis using an anti-His tag antibody.

EMSA and DNase footprinting

Electrophoretic mobility shift assay (EMSA) was performed according to manufacturer's instructions (Promega Gel Shift Assay System). Full length Sia protein-DNA complexes were resolved on a 5% native polyacrylamide gel in 0.25X Tris-Borate-EDTA buffer for one hour at 240V. Sia and Twn homeodomain (HD) fragments, complexes were resolved on an 8% native polyacrylamide gel. Stability of protein-DNA complexes for wild-type and mutated probes was determined by addition of a 100-fold molar excess of cold unlabeled wild-type oligonucleotide as a competitor after the initial binding reaction. The bound complex was collected at specific time points, resolved by EMSA, and protein-DNA complex formation was quantified using the ImageQuant program (Molecular Dynamics). For heterodimerization of Sia and Twn when bound to DNA, EMSA was performed with increasing concentrations of His-Sia112-215 and constant concentration of Twn HD. DNase footprinting was performed according to standard procedures (Brenowitz et al., 2001). End labeled DNA was incubated with 0.5 – 2.0µg recombinant Sia or Twn protein. Upon completion of DNase cleavage, DNA was extracted with phenol/chloroform,

ethanol precipitated and radiolabelled DNA fragments were resolved on a 6% denaturing polyacrylamide gel.

Luciferase reporter assay

One-cell stage *Xenopus* embryos were injected in the animal pole with *in vitro* transcribed mRNA encoding the indicated proteins. At the two-cell stage, one blastomere was injected with 100pg of pGL3-Gsc-Luciferase containing the wild-type or mutated -226Gsc promoter in combination with 10pg of pGL3-CMV-*Renilla* as an internal control (*Renilla* luciferase under the control of the constitutive CMV promoter) (Kessler, 1997). Animal pole explants prepared at the blastula stage were collected at midgastrula stage and luciferase activity was determined using the Dual Luciferase Assay Kit (Promega) on a TD-20/20 luminometer (Turner Designs). Error bars represent standard error of at least three independent experiments.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described (Blythe et al., 2009). One-cell embryos were injected with 50pg of myc-Sia mRNA, 50pg of myc-Twn mRNA, 250pg of myc-FoxH1 mRNA or 50pg of Xnr1 mRNA. An average of 75 embryos were collected at stage 10.25 and processed for ChIP. Polyclonal anti-myc antibody (Millipore cat# 06-549) or anti-Smad2/3 (Millipore cat#07-408) was used for immunoprecipitation. As a control for IP of endogenous Smad2/3, rabbit IGG (Calbiochem cat #NI01) was used. Sequential chromatin immunoprecipitation was performed as described (Geisberg and Struhl, 2004) with two immunoprecipitations using polyclonal anti-myc antibody (Millipore, 06-549) and anti-GST antibody (GE Lifesciences, 27-4577-01). Briefly, 150pg of mRNA encoding differentially tagged (either GST or myc) Sia or Twn was injected into one-cell embryos. An average of 75 embryos was collected at stage 10.25 and processed for ChIP. The eluate from the first immunoprecipitation was subdivided, with half processed for ChIP and half used for the second immunoprecipitation. The second immunoprecipitation was performed by adding 1.4ml of RIPA

buffer to 100μl of eluate, and addition of the second antibody according to the ChIP protocol. Quantitative PCR was performed using primers specific for *Gsc*, *Ef1α* or *Xmlc2* as previously described (Blythe et al., 2009). Primers for QPCR amplification of the Cer promoter are F – 5'-GGAACAGCAAGTCGCTCAGAAACA-3' and R – 5'-CTCCATCATTCACAAGGCAGACGA-3'. Primers for QPCR amplification of the Chd promoter are F – 5'-GCTGAGTCAGGATGCTGTTTCTGAGT-3' and R – 5'-TGCCCAAGGAAAGTGTCTCTTAACCG-3'.

In situ hybridization and histology

For whole mount in situ hybridization, embryos were fixed and hybridized with antisense digoxygenin-labeled RNA probes as described (Sive et al., 2000). For whole mount in situ hybridization of bisected embryos, embryos were fixed in MEMFA and bisected in a 30% sucrose/PBS solution. Hybridized probe was detected using alkaline phosphatase-conjugated anti-digoxygenin Fab fragments (Roche) and BMpurple (Roche) as a substrate for color development. Antisense probes were synthesized from linearized plasmid DNA using the Megascript kit (Ambion) supplemented with 2mM digoxygenin-11-UTP (Roche). Templates for in situ probes were pCS2+Sia (this study), pCS2+Twn (this study), pCS2+FoxH1 (Fast1) (Yaklichkin et al., 2007), pCS2+Gsc (Yao and Kessler, 2001), pCS2+Chd (Sasai et al., 1994), pGEM-Xbra (Wilson and Melton, 1994), pBS-Opl (Kuo et al., 1998), pCS2+Cer (Bouwmeester et al., 1996), and pGEM-XWnt8 (Sokol et al., 1991). Antibody staining for total Smad2/3 (Millipore cat#07-408) on bisected embryos was carried out as described (Sive et al., 2000). For histology, 10μm sections were prepared from paraplast-embedded embryos and dewaxed sections were stained with Hematoxylin/Eosin before coverslipping with Permount as previously described (Sive et al., 2000).

Morpholino oligonucleotides

The Sia and Twn morpholino antisense oligonucleotides (Sia MO and Twn MO) are complementary to nucleotides of 1-25 of *Xenopus* Sia (5'-GCTCCATTTCAGCCTCATAGGTCAT -3') and nucleotides 1-25 of *Xenopus* Twin (5'-GCTCAAGTTCAGAGTCACAAGTCAT-3') (Gene Tools). Individual or mixed oligonucleotides were injected at a total dose of 50ng per embryo. As a control, embryos were injected with equal doses of the standard control morpholino (5'-CCTCTTACCTCAGTTACAATTTATA-3') (Gene Tools).

Reverse Transcription – Polymerase Chain Reaction

For RT-PCR analysis, total RNA was isolated using TRIzol (Invitrogen), and cDNA synthesis was performed as described (Wilson and Melton, 1994). cDNAs were amplified using quantitative PCR. PCR primers for amplification of Gsc transcript were F – 5'-CCTCTGGAATAAGAATAAAGACTTGCAC-3' and R – 5'-CTCTATGTACAGATCCCACATCGT-3'. PCR primers for amplification of *Cer* transcript were F – 5'-CTGAACCACCTGACGCTAATTGT-3' and R – 5'-CTGTGCAGTTTGGTGGAAGTTGCT-3'. PCR primers for amplification of *Chd* transcript were F – 5'-CAGCTGCAAAAACATCAAACA-3' and R – 5'-CAAGTCTTGCAGCAATGTCC-3' (Skirkanich and Klein, unpublished data). The primers for amplification of *Ef1*α transcript were previously described (Agius et al., 2000).

Western blotting

One-cell stage embryos were injected with the indicated *in vitro* transcribed RNA For standard Western analysis, embryos were lysed (10 μl/embryo) in 0.1 M Tris-HCl, pH 6.8, supplemented with protease inhibitors. Extracts were cleared by centrifugation, and half an embryo equivalent was loaded per well. An anti- myc polyclonal antibody (Millipore) was used at a 1:1000 dilution and was detected with a 1:3000 dilution of peroxidase-coupled secondary antibody by chemiluminescence (Amersham Biosciences).

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