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Coordination of patterning and morphogenesis during early development in *Xenopus laevis*

by

Cameron Ruth Thompson Exner

A dissertation submitted in partial satisfaction of the requirements for the degree of

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in

Molecular & Cell Biology

in the

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of the

University of California, Berkeley

Committee in charge:

Professor Richard M. Harland, Chair
Professor Matthew D. Welch
Professor Craig T. Miller
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Abstract

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Over the course of development, cells and tissues of the embryo must take on the correct fates and morphologies to produce a functioning organism. The patterning events and morphogenetic processes that accomplish this task have been the subject of decades of research, the consequence of which has been a detailed comprehension of the molecular mechanisms that regulate each. Equally important is an understanding of the mechanisms that coordinate patterning with morphogenesis, such that they occur with the correct relative spatiotemporal dynamics. My thesis work sought to characterize such co-regulation in the context of two developmental events in a vertebrate model, the African clawed frog *Xenopus laevis*: induction of bottle cell formation at the onset of gastrulation after germ layer induction, and regulation of the morphogenetic movements of neurulation in relation to neural plate patterning.

Chapter 1 of this dissertation provides a general introduction to the patterning and morphogenetic events of early development relevant to my thesis. Chapter 2 presents a discussion of my work to characterize the function of two signaling pathways, namely Nodal signaling and Wnt/Planar Cell Polarity, in the induction of bottle cells. My experiments confirm the requirement for Nodal signaling in bottle cell induction, but do not support a role for the individual transcriptional targets of Nodal signaling tested here or for Wnt/PCP. Chapter 3 summarizes my work to address the function of two transcription factor-encoding genes, *sall1* and *sall4*, in neural development, including their roles in anteroposterior neural patterning, neural tube morphogenesis, and neural differentiation. My work shows that both *sall1* and *sall4* are required for all three processes, and supports the hypothesis that their key role in this context is to transcriptionally repress stem cell factors of the *pou5f3* family, allowing progression through neural development. As a whole, this work summarizes my research to characterize molecules that co-regulate early patterning and morphogenetic events in the *X. laevis* embryo.
Dedication

This dissertation is dedicated to my parents, Claudia R. Thompson and George R. Exner, and to my sister, Laurel E. E. Thompson.
Acknowledgments

I thank Richard Harland and the members of the Harland lab for years of support, training, camaraderie, and love for developmental biology. Special thanks go to Edivinia Pangilinan, a dear friend without whom the lab could not function, and to James Evans, who runs the frog facility with expertise and geniality. I also extend my deepest thanks and warmest wishes to the three remarkable students who have contributed to this work under my mentorship: Albert Kim, Caroline Creasey, and Sarah Mardjuki. Finally, I thank my committee members and John Gerhart, who have provided me with excellent mentorship complementing what I have received from my own lab.
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Chapter 1: Introduction

1.1 Coordination of patterning and morphogenesis during development

Over the course of vertebrate development, a single cell is transformed into an organism of complex function. At every stage, embryonic cells and tissues face two major tasks: adopting the correct identity and acquiring the correct shape. Specification of fates is achieved through patterning events, for example via cell-cell signaling interactions and transcriptional regulation; generation of shape is accomplished through morphogenesis, for example by cell shape changes or rearrangements. Without these two core processes, no functional cell types could be produced in any configuration supportive of further development or survival. Furthermore, if either process fails at a particular developmental stage, resulting defects can have far-reaching consequences for the organism. It is therefore of critical interest to understand how patterning and morphogenesis are robustly regulated and coordinated during development. My thesis work has focused on two instances of this kind of coordination, one concerning germ layer induction and gastrulation and the other concerning neural patterning and morphogenesis.

Although patterning and morphogenetic events in the embryo are intimately linked, they are conceptually separable. As a simple hypothetical example, a sheet of equivalent cells in which all cells are dividing randomly could undergo thickening or extension, without a patterning event to confer asymmetry of fates within that sheet. Conversely, a sheet of quiescent cells could be patterned along one or more axes by the influence of neighboring signaling centers without undergoing any shape change whatsoever. Alternatively, a sheet might be induced by the same signal(s) to both change shape and acquire different fates along its axes. The molecular pathways that drive concurrent patterning and morphogenetic events may overlap extensively, or might be distinct. Either way, insight into the nature of patterning and morphogenetic regulatory mechanisms constitutes a significant step towards a fundamental understanding of how biological systems achieve a fully formed and functional state through development.

A long history of efforts in the field of developmental biology has sought to characterize both patterning and morphogenesis, using techniques ranging from classical embryology to next-generation sequencing. A sizeable body of literature catalogs what we have learned about individual patterning and morphogenetic events and the molecular underpinnings thereof. Much of the work investigating early developmental stages has been done in amphibian embryos, due to their large size, ready accessibility, and robustness to manipulation. Experiments in the African clawed frog, *Xenopus laevis*, enabled construction of lineage maps, careful description of morphogenesis, and identification of signaling centers responsible for establishment of the three germ layers and embryonic axes (Spemann and Mangold, 1924; Nieuwkoop, 1969; reviewed in Hamburger, 1988; Niehrs, 2004). In more recent years, the field has gained a deeper understanding of these interactions at the genetic and molecular level. A multitude of signaling pathways, transcription
factors, cytoskeletal elements, and other players have been implicated in various events during embryogenesis in the frog, and many of these have conserved roles in vertebrate development in general (see below; reviewed in Kimelman et al., 1992; Dawid, 1994; Harland and Gerhart, 1997; Heasman, 1997; De Robertis et al., 2000; Kimelman, 2006; Heasman, 2006). A brief introduction to early development follows, with emphasis on events particularly relevant to my thesis work.

During early development, the fertilized egg divides rapidly to produce several thousand daughter cells; these will subsequently signal to each other and execute autonomous behaviors to give rise to all tissues of the embryo. At the mid-blastula stage, these cells are all pluripotent (Heasman, 1994), and will become specified and committed based on both maternal components they have inherited and signals they receive from other regions of the embryo.

Expression of factors that inhibit differentiation is critical for maintaining and amplifying pluripotent precursor populations during these early stages (Morrison and Brickman, 2006; Livigni and Brickman, 2013), as is true in other vertebrate embryos (Nichols et al., 1998; Niwa et al., 2000; Niwa et al., 2002; Loh et al., 2006; reviewed in Jerabek et al., 2014; Radzisheuskaya and Silva, 2014; Wu and Schöler, 2014). Pluripotency is enforced by a network of conserved transcription factors including oct3/4. These factors are expressed broadly early on and inhibit the expression of differentiation genes; functional depletion in *Xenopus* results in precocious differentiation (Frank and Harland, 1992; Whitfield et al., 1993; Henig et al., 1998; Cao et al., 2004; Cao et al., 2006; Cao et al., 2007). While these genes are absolutely required for development, especially during early stages in the maintenance of undifferentiated precursors, it is equally crucial to turn off these programs later in development to allow those precursors to progress down their correct differentiation paths and generate mature, functional cell types (Snir et al., 2006; Archer et al., 2011; Rousso et al., 2011).

The pluripotent cells of the blastula begin to acquire different fates at the mid-blastula stage and undergo drastic rearrangements as gastrulation begins. My thesis work involved two projects that sought to address how patterning and morphogenesis are coordinated in two specific cases, as described in more detail below: 1) bottle cell formation at the onset of gastrulation after germ layer induction, and 2) neurulation and anteroposterior patterning of the neural plate. As part of the latter, I also began to assess how regulation of differentiation influences patterning and morphogenesis.

### 1.2 Bottle cell formation at the onset of gastrulation after germ layer induction

The fertilized egg undergoes serial divisions to produce the blastula, a roughly spherical structure of approximately 4,000 cells with a hollow blastocoel.
At this stage, maternally deposited components in the vegetal hemisphere and marginal zone have initiated transcription of key zygotic genes in the first steps of germ layer induction. More specifically, maternal VegT activates transcription of Xnr genes, which encode Nodal proteins of the TGF-β family of signaling ligands (Kofron et al., 1999). These Nodal proteins, along with a few other ligands such as Derriere, are secreted by vegetal and marginal zone cells and activate both autocrine and paracrine signaling through their receptors. Upon ligand binding and activation, these receptors phosphorylate Smad2/3, which subsequently binds Smad4; this complex enters the nucleus and regulates the transcription of target genes (Germain et al., 2000). In vegetal cells that also contain maternal VegT, endodermal gene expression is activated both directly by VegT (Chang and Hemmati-Brivanlou, 2000) and as a consequence of Nodal signaling (Zhang et al., 1998; Kofron et al., 1999). In cells further from the vegetal pole, which lack VegT, Nodal signaling induces mesodermal gene expression (Kofron et al., 1999). By virtue of its physical distance from the vegetal pole and in conjunction with localized maternal components, the animal portion of the embryo does not express VegT and does not receive Nodal signaling; this tissue also expresses factors that restrict the response to mesoderm-inducing signals (Dupont et al. 2005, Sasai et al., 2008; reviewed in Piccolo 2008), and thus adopts ectodermal fate. In this way, maternally deposited components and zygotically expressed signaling molecules induce the three germ layers of the vertebrate embryo (reviewed in De Robertis et al., 2000). These three germ layers will give rise to all tissues of the embryo and later adult: endoderm to the gastrointestinal tract and other derivatives, such as the lungs; mesoderm to muscle, bone, and connective tissue; and ectoderm to epidermis and neural tissues.

What has not yet been accomplished at this stage, however, is arrangement of these germ layers into a conformation that can generate a functional embryo. At the blastula stage, part of the endoderm and mesoderm remain on the surface of the roughly spherical embryo, and the ectoderm covers only the animal portion. It is lethal if the germ layers differentiate into their respective derivatives in this spatial arrangement, as they do in an exogastrula: for example, the gut would remain external, and not be covered in muscle or skin. As a less visually dramatic but equally important example, failure to reorganize even before terminal differentiation would prevent embryonic tissues from interacting in important ways; for example, the dorsal mesoderm and ectoderm must come into apposition for anteroposterior patterning to occur correctly (reviewed in Stern, 2005; Ozair et al., 2013). To organize all of its germ layers into a conformation compatible with continued development, the embryo undergoes the suite of morphogenetic movements that comprise gastrulation. In short, the endoderm and mesoderm involute and move towards the interior of the embryo, while the ectoderm spreads to cover the embryo’s surface. These movements have been detailed at length (reviewed in Locascio and Nieto, 2001; Keller et al., 2003; Keller, 2005; Solnica-Krezel, 2005; Solnica-Krezel and Sepich, 2012). However, particularly relevant for the work described here is the first outwardly visible step of gastrulation, the formation of bottle cells.
Bottle cells are a small population of morphogenetically defined endodermal cells that change shape to direct subsequent movements of the endomesoderm towards the interior of the embryo (Keller et al., 1985; Hardin and Keller, 1988). Bottle cells first form on the dorsal side of the embryo, where Wnt/β-catenin signaling and elevated levels of Nodal have established Spemann’s organizer (Heasman, 1994; Watabe et al., 1995; Larabell et al., 1997; Laurent et al., 1997; Nishita et al., 2000; reviewed in Harland and Gerhart, 1997) and later form laterally and then ventrally to compose the blastopore ring. Each cell undergoes apical constriction to change from a columnar or cuboidal shape to a wedge or "bottle" shape; this change is driven by the contractile activity of an apical actomyosin network, and requires intact microtubules and endocytosis of the apical membrane (Lee and Harland, 2007; Lee and Harland, 2010). Individual bottle cells each undergo such apical constriction while remaining tightly connected their neighbors through adherens junctions. The consequence is to bend the tissue as a whole. Importantly, because the shrinking apical membranes of these cells face the exterior of the embryo, the result is to bend the tissue inwards. Thus the surface and its connected cells invaginate the endomesodermal tissues. In fact, if bottle cells fail to form, the embryo often extends its endomesoderm towards the exterior, forming an exogastrula (Keller, 1986; Keller and Danilchik, 1988).

Although the phenomenon of bottle cell formation has been thoroughly documented, and some of the mechanism of formation (e.g., actomyosin contractility) described, it remains unclear what induces bottle cell formation in such a spatially restricted part of the embryo. This must also be a temporally regulated process, to ensure that the three germ layers are rearranged appropriately just after germ layer establishment and before the layers begin to differentiate or signal to each other inappropriately. It is critical to understand how morphogenetic events such as bottle cell formation are coordinated with patterning events like germ layer induction. The goal of my work so far has thus been to identify molecules and define pathways that induce and regulate bottle cell formation at the onset of gastrulation.

Previous work has approached this question to a degree. Because of their major role in germ layer induction and their expression in or near presumptive bottle cells, early candidate bottle cell inducers were TGF-β signaling molecules. Gain- and loss-of-function experiments have shown that Nodal proteins, but not other TGF-β ligands (e.g., Activin), are both required and sufficient for endogenous bottle cell induction (Agius et al., 2000; Kurth and Hausen, 2000). However, Nodals are broadly expressed throughout the endomesoderm, so how they might induce bottle cells in a very restricted manner has remained unclear. Furthermore, it has not been shown that Nodal signaling, through some set of transcriptional events or other cell-autonomous mechanism, directly induces bottle cell formation. Figure 1.1 illustrates the basic concepts of germ layer induction and bottle cell formation downstream of Nodal signaling. The only other secreted signaling ligand shown so far to be required and sufficient for bottle cell formation is Wnt5a (Choi and Sokol,
a noncanonical Wnt known to activate Wnt/Planar Cell Polarity (Wnt/PCP) signaling to drive later morphogenetic movements (Moon et al., 1993; Wallingford et al., 2001; Oishi et al., 2003; Schambony and Wedlich, 2007; Qian et al., 2007; Cha et al., 2008). As is true for Nodal, a direct induction of bottle cell formation by Wnt5a has not yet been demonstrated. The PCP components Vangl2 and Daam1 and the intracellular apicobasal polarity protein Lgl were also published to be required and sufficient for bottle cell formation, but a direct mechanism has not been demonstrated (Choi and Sokol, 2009; Ossipova et al., 2015b).

1.3 Regulation of neural patterning, morphogenesis, and differentiation by \textit{sall1} and \textit{sall4}

After gastrulation, the three germ layers have been arranged appropriately for development to proceed. Major signaling events during and after gastrulation continue to pattern each germ layer. Crucially, BMP antagonists secreted by the organizer mesoderm have established a BMP-free zone on the dorsal side. The dorsal ectoderm consequently adopts a neural fate in a dorsal territory, whereas the lateral and ventral ectoderm do experience BMP signaling and are induced to become epidermis. Analogous BMP signaling and antagonism in the mesoderm and endoderm occur, and the dorsal-ventral axis of the embryo is thus established (Wilson and Hemmati-Brivanlou, 1995; Suzuki et al., 1997; Schmidt et al., 1995; Sasai et al., 1994; Sasai et al., 1995; Hawley et al., 1995; Smith and Harland, 1992; Hemmati-Brivanlou et al., 1994; Khokha et al., 2005; reviewed in Stern, 2005; Ozair et al., 2013).

Gastrulation movements reposition tissues to have additional and equally critical interactions to pattern the other major embryonic axis, the anterior-posterior axis. Initially circumblastoporal in expression, Wnt is subsequently expressed in the posterior mesoderm, and activates canonical signaling through β-catenin in a graded fashion (Kiecker and Niehrs, 2001). High levels of signaling activity here induce posterior fates in receiving cells; for example, the neural tissue is induced to become spinal cord. In the anterior, low levels of Wnt signaling are maintained by the secretion of Wnt antagonists by the anterior organizer mesoderm, or head organizer; as a consequence, neural tissue here adopts an anterior fate, e.g., forebrain. The role of Wnt signaling and its antagonism in anteroposterior patterning have been well documented (McGrew et al., 1995; Bouwmeester et al., 1996; Glinka et al., 1998; Piccolo et al., 1999; Domingos et al., 2001; Kiecker and Niehrs, 2001). In summary, the dorsal ectoderm has been patterned in two steps, as proposed by Nieuwkoop (Nieuwkoop, 1952a; Nieuwkoop, 1952b; Nieuwkoop, 1952c): first, antagonism of BMP signaling to establish the neural plate on the dorsal side; and second, graded Wnt signaling to induce different fates along the anterior-posterior axis. In addition to Wnt signaling, FGF and retinoic acid (RA) signaling also contribute to posteriorization of the neural plate (Durston et al., 1989; Cox and Hemmati-Brivanlou, 1995; Niehrs, 1999; Maden,
2006; reviewed in Stern, 2005; Ozair et al., 2013). How these pathways interact to induce posterior identity is an area of active investigation.

While it has been firmly established that Wnt signaling is required and sufficient to induce posterior fates in the neural plate, the molecular mechanisms of this posterior induction have not been thoroughly analyzed. With few exceptions, the targets of Wnt/β-catenin signaling that actually effect posteriorization have not been characterized. Previous work in the lab identified putative direct targets of Wnt/β-catenin signaling, and began to investigate the roles of these genes in neural patterning (Young et al., 2014). Two target genes in particular, sall1 and sall4, stood out as interesting candidates for inducers of posterior identity. These two genes encode Zinc-finger transcription factors that can act as repressors or activators (Zhang et al., 2006; Lim et al., 2008; Lauberth et al., 2007; Yang et al., 2012; reviewed in de Celis and Barrio, 2009; Sweetman and Münsterberg, 2006), and so may regulate neural patterning at the transcriptional level. As would be predicted for direct targets of canonical Wnt signaling, they are expressed in the posterior neural ectoderm, in the correct location to have the proposed role downstream of Wnt. Furthermore, our lab has shown using a loss-of-function approach that sall4 is required for posterior induction. It is also required for neural differentiation later in development. When sall4 function is lost, genes of the pou5f3 family are derepressed, and these stem cell factors hold the cells of the neural plate in an undifferentiated state. Whether sall1 is also required for neural patterning or differentiation remained untested.

In addition to patterning along the anterior-posterior axis during these stages, the neural tissue undergoes morphogenetic movements to transform a flat plate on the exterior of the embryo to an interior tube (Smith and Schoenwolf, 1997; Schoenwolf, 1991; reviewed in Wallingford, 2005). This neural tube, running from the anterior to the posterior on the dorsal side, will give rise to the central nervous system (i.e., brain and spinal cord) of the organism. The details of neural tube morphogenesis, neurulation, have been an area of interest for developmental biologists for decades. In particular, two important cell behaviors have been shown to drive neural tube closure. First, medial and dorsolateral populations of cells called hinge point cells form by apical constriction. This cell shape change begins to fold the neural plate, generating kinks that elevate the left and right neural folds (Schoenwolf and Franks, 1984; Lawson et al., 2001; Colas and Schoenwolf, 2001). Simultaneously, convergent extension movements in the neural plate and underlying mesoderm elongate the embryo from anterior to posterior while narrowing it in the orthogonal left-right axis (Keller et al., 1992; Keller et al., 2008b). This brings the elevating neural folds closer together, such that they can subsequently fuse at the midline to complete neural tube closure. Some regulators of these behaviors have been characterized. For example, shrm3 is required to recruit the active actomyosin network that drives hinge point cell formation (Hildebrand and Soriano, 1999; Haigo et al., 2003), and Wnt/Planar Cell Polarity signaling is required to orient and execute convergent extension movements (Sokol, 1996; Tada and Smith, 2000; Wallingford et al., 2000; Habas et al., 2001b; Darken et
As mentioned above, pluripotency factors are expressed early in development, and must be downregulated to allow differentiation at later stages, including during neurulation. In *Xenopus*, the endogenous *oct4* homologues of the *pou5f3* family are expressed during gastrulation and downregulated by late neurula stages (Frank and Harland, 1992), and overexpression and knockdown each cause defects in neural patterning or differentiation (Morrison and Brickman, 2006; Snir et al., 2006; Archer et al., 2011). Thus, characterization of neural development requires attention to the regulation of differentiation, as well as to patterning and morphogenesis.

As for bottle cell formation after germ layer induction, it is likely important that neural patterning and neural tube morphogenesis occur with the correct relative spatiotemporal dynamics. For instance, a completely patterned neural plate that does not undergo tube morphogenesis would be left on the embryo’s exterior; subsequent differentiation into neurons may occur, but the tissue would fail to undergo proper dorsal-ventral patterning, attain functional brain and spinal cord morphology, innervate the correct target tissues, etc. Thus, it is not unreasonable to expect that regulatory mechanisms exist to coordinate neural patterning and morphogenesis with each other and with differentiation. The basic concepts of potential coordination between neural patterning downstream of Wnt and neural tube formation are illustrated in Figure 1.2. A primary goal of my work has been to address what the connection may be between regulators of patterning and regulators of morphogenesis in this context.

To begin identifying such regulators, I turned to candidates from our group’s screen for direct Wnt targets. In addition to the published patterning and differentiation defects caused by loss of *sall4* function, described above, our group noticed associated morphogenetic problems. More specifically, *sall4*-deficient embryos exhibit neural tube closure defects. Upon examination of the *sall1* loss-of-function phenotype, I discovered that *sall1* is also required for neural tube closure. Morphogenetic defects observed upon loss of *sall1* or *sall4* function are caused in part by a failure in hingepoint cell formation. Additionally, I showed that *sall1* is required for neural patterning and differentiation. Like *sall4*, *sall1* represses *pou5f3* family gene expression in the neural plate to allow neural development to proceed. Thus, due to their regulation of differentiation, *sall1* and *sall4* constitute regulators of both anterior-posterior neural patterning and neural tube morphogenesis.

### 1.4 Summary of thesis

At the core, my graduate work was motivated by the observation that patterning and morphogenesis occur together in space and time, and the question of whether they are co-regulated to ensure their coordination. There are many
reasons to expect that they are indeed co-regulated and that such coupling may be indispensible for development. First, cells and tissues with an appropriate fate in terms of gene expression but without the correct morphology or physical position in the embryo may be unable to execute their functions; conversely, cells with a particular morphology or location will not function if they do not have the correct identity. Second, movements often bring into apposition tissues that will subsequently signal to each other to inform the next steps of their patterning; likewise, patterning events can initiate signaling that drives morphogenesis. Because they are so intertwined in every developmental process, “fate” and “morphology” of a cell can be difficult to separate entirely. However, the processes that induce gene expression governing identity and the processes that generate morphology allowing function are conceptually distinguishable; I refer to these processes here as “patterning” and “morphogenesis.”

My thesis work sought to address how patterning and morphogenesis are coupled during two developmental processes in Xenopus: 1) formation of bottle cells at the onset of gastrulation after germ layer induction, and 2) neural tube closure in conjunction with anteroposterior neural patterning. I have made use of candidate and unbiased approaches in an attempt to identify genes that regulate patterning, morphogenesis, or both in these contexts. Gain- and loss-of-function experiments were performed to test the activity of possible co-regulators.

My investigation of bottle cell formation began with the goal of identifying bottle cell inducers beyond the master regulator, Nodal signaling, and a few additional published inducers, including the noncanonical Wnt/PCP ligand Wnt5a and the apicobasal polarity protein Lgl. Select Nodal targets (Kim et al., 2011) were not sufficient to induce ectopic apical constriction when overexpressed, and my work was unable to provide a link between Nodal signaling and a direct bottle cell inducer. My data could not reproduce published experiments supporting Wnt5a, Vangl, or Lgl’s function as bottle cell inducers (Choi and Sokol, 2009; Ossipova et al., 2015b). In addition, other members of the Wnt/PCP pathway were neither required nor sufficient to induce bottle cells. Thus, despite efforts to identify a direct bottle cell inducer, such a molecule remains elusive.

My work on neural tube development involved the further characterization of two transcription factor-encoding genes, sall1 and sall4. Our group has previously shown that sall4 is required for posterior neural fate induction (Young et al., 2014). I showed that sall1 is also required for posterior patterning, and that both are required for neural tube closure and neural differentiation. The neural tube closure defects observed in morphants are likely due to failure of apical actin localization in neural plate cells and failure of hingepoint cell formation; a connection between transcriptional regulation by the sall genes and regulators of the cytoskeleton and cell shape has not yet been identified. As previously shown for sall4, sall1 is also required to repress expression of the pou5f3 gene family, the Xenopus homologs of the mammalian stem cell factor oct4. We have devised a model in which sall1 and sall4 repress pou5f3 genes to allow differentiation, upon
which neural patterning and neural tube morphogenesis are allowed to proceed. In this way, \textit{sall1} and \textit{sall4} coordinate neural patterning and morphogenesis by releasing the metaphorical breaks on both processes during neural development.
Figure 1.1 Germ layer and bottle cell induction downstream of Nodal

Nodal signaling (orange) patterns the embryo, inducing endomesoderm (yellow and red) during blastula stages. This pathway also induces the formation of bottle cells (green), which direct subsequent morphogenetic movements to arrange the germ layers of the gastrulating embryo. The molecular mechanisms directly linking Nodal signaling and bottle cell formation have not yet been identified. For simplicity, other regulators of germ layer identity and/or bottle cell formation are not shown. Lateral view; animal pole up, vegetal pole down.
Figure 1.2 Anteroposterior patterning of the neural plate and morphogenesis of the neural tube

Wnt signaling (indigo) and other pathways pattern the embryo along the head-to-tail axis. The neural plate is shaped through the morphogenetic movements of neurulation, driven in part by apical constriction to form hinge point cells (green), to form the neural tube. Molecules downstream of Wnt and other signaling pathways may impinge on morphogenetic events, including bottle cell formation. This tissue will give rise to the central nervous system. For simplicity, other regulators of neural patterning and morphogenesis are not shown. Posterio-lateral and transverse cross-sectional view; anterior to the upper right.
Chapter 2: Characterization of direct inducers of bottle cell formation

2.1 Introduction

Gastrulation in the vertebrate embryo is a suite of highly coordinated morphogenetic movements that result in the internalization of the mesodermal and endodermal precursors and leave ectoderm covering the exterior surface (reviewed in Solnica-Krezel, 2005; Solnica-Krezel and Sepich, 2012). The first externally visible sign of gastrulation in amphibians is the formation of bottle cells, which direct invagination and subsequent involution towards the interior of the embryo (Rhumbler, 1902; Baker, 1965; Keller et al., 1985; Hardin and Keller, 1988). The bottle cells constitute a morphogenetically defined population of endodermal cells that form around the circumference of the blastopore, first forming on the dorsal side and later laterally and ventrally to complete the blastopore ring. Each individual bottle cell undergoes a cell shape change known as apical constriction, converting from a cuboidal or columnar shape to a “wedge” or “bottle” shape due to the action of actomyosin contractility on the apical (in this case, exterior-facing) side of the cell (Lee and Harland, 2007). As neighboring bottle cells execute this behavior together, maintaining contacts and transmitting force through cell-cell junctions, the tissue bends and invaginates inward. Bottle cells also exhibit invasive and migratory behaviors away from the exterior (Holtfreter, 1943). In this manner, individual cell shape changes sum to direct subsequent gastrulation movements towards the interior. These cells are not strictly required for \textit{Xenopus} gastrulation to occur (Keller, 1981), but failure of bottle cells to apically constrict can cause the mesoderm and endoderm to extend away from the embryo towards the exterior, the lethal phenomenon of exogastrulation (Keller, 1986; Keller and Danilchik, 1988).

Although not always termed bottle cells, analogous apically constricting cells perform a similar role at the onset of gastrulation in other vertebrates and in invertebrates. For example, cells undergo an epithelial-to-mesenchymal transition (EMT) via apical constriction and ingress at the primitive streak in chicken and in mouse (Viebahn, 1995; Kimelman, 2006; Voiculescu et al., 2007). In contrast to bottle cells, these ingressing cells do not maintain their contacts with neighbors. Primary invagination during sea urchin gastrulation is also driven by apical constriction (Kimberly and Hardin, 1998; Nakajima and Burke, 1996). In the fly, mesoderm cells apically constrict to cause invagination of the ventral furrow (Sweeton et al., 1991; Costa et al., 1994). Endodermal precursors in the nematode embryo initiate their own ingestion via apical constriction (Lee and Goldstein, 2003; Nance and Priess, 2002; Marston et al., 2016). This cell shape change has also been shown to initiate gastrulation in cnidarians (Magie et al., 2007; Kraus and Technau, 2006). Thus, apical constriction is a conserved mechanism involved in gastrulation throughout metazoans. More broadly, apical constriction contributes to other instances of epithelial morphogenesis, including tube formation during vertebrate neural tube closure and \textit{Drosophila} trachea and salivary gland morphogenesis (Broda and Casanova, 2006; Nikolaidou and Barrett, 2004; Xu et al., 2008); organogenesis, including lens formation in vertebrates and \textit{Drosophila} eye
furrow formation (Ready et al., 1976; Tomlinson, 1985); wound healing (Davidson et al., 2002); and other EMT events, such as during cancer metastasis (Rao and Li, 2004; Yang et al., 2004; reviewed in Yang and Weinberg, 2008; Sánchez-Tilló et al., 2012).

Apical constriction, then, is a widespread cell shape change involved in the rearrangement of a variety of tissues during development and homeostasis (reviewed in Martin and Goldstein, 2014). This raises the question of whether it is regulated similarly in each context in which it is employed, or whether different mechanisms exist for each case. Over decades of research to address this question, two themes have emerged: (1) whereas the force-generating machinery required for apical constriction, particularly actin and activated myosin, appears to be conserved, (2) other regulators seem to vary widely. In Drosophila, signaling through Fog and Concertina is required to coordinate apical constriction during ventral furrow formation and dorsal closure (Manning et al., 2013; Dawes-Hoang et al., 2005; Azevedo et al., 2011). In C. elegans, endodermal GATA transcription factors, apicobasally polarized PAR proteins, and Wnt/β-catenin signaling are all involved in endodermal precursor apical constriction and ingestion (Nance et al., 2003; Lee et al., 2006). In vertebrates, Shroom3 and Nectin-2 known to be required for hingepoint cell formation via apical constriction during neural tube morphogenesis, and they are both sufficient to induce ectopic apical constriction in a naïve epithelium (Haigo et al., 2003; Lee et al., 2009). Shroom3 localizes to the apical surface, and recruits actin and activators of myosin to drive apical constriction. Characterization of these many regulators has highlighted that diverse mechanisms converge on a small set of cytoskeletal players to induce apical constriction in different contexts.

In many cases, direct inducers of apical constriction have not been identified. Bottle cells constitute such an example, in that they have been shown not to require any of the regulators mentioned above, including Shroom3, which they do not express. As such, some other mechanism must induce the cytoskeletal activities that drive bottle cell formation. Early on in the search for bottle cell inducers, developmental biologists turned to components of the Nodal signaling pathway as candidates. Nodal signaling is initiated in the endoderm by maternal factors (see Chapter 1), and is required for endodermal identity and for mesoderm induction. Critically, Nodal signaling was shown to be required for endogenous bottle cell formation and sufficient to induce ectopic apical constriction (Lustig et al., 1996; Baker and Harland, 1996; Agius et al., 2000; Kurth and Hausen, 2000). This is consistent with, and likely underlies, the spatiotemporal proximity of germ layer induction and bottle cell formation (see Figure 1.1; (Smith and Howard, 1992; Kurth and Hausen, 2000). The demonstrated role for Nodal suggests the intuitively appealing hypothesis that transcriptional targets of this signaling pathway are activated in presumptive bottle cells and cell-autonomously induce the apical constriction that defines this population. However, the full set of direct transcriptional targets of Nodal signaling in this tissue had not yet been identified. Just before I began my thesis work, a collaborator performed a screen for Nodal
targets (Kim et al., 2011). From the resulting list, I chose several candidates strongly responsive to Nodal manipulation to investigate as potential bottle cell inducers.

One research group has demonstrated that certain molecules normally associated with Planar Cell Polarity (PCP) signaling are also required for bottle cell formation in *Xenopus* (Choi and Sokol, 2009; Ossipova et al., 2015b). Initially, Wnt5a was implicated; more recent work has described roles for Vangl2 (*strabismus*) and Daam1. These publications also argue that all three are sufficient to induce ectopic apical constriction. Bottle cells have not been shown to be planar polarized; instead, these three molecules appear to regulate aspects of apicobasal polarity in this context. The same laboratory showed that the apicobasal polarity regulator Lgl is also required and sufficient downstream of Activin and Wnt5a for bottle cell formation and ectopic apical constriction (Dollar et al., 2005; Choi and Sokol, 2009). I attempted to reproduce the finding that these molecules are required for bottle cell apical constriction, and tested other components of the Wnt/PCP pathway to determine whether they are also involved.

### 2.2 Results

**Ectopic TGF-β signaling is sufficient to induce ectopic bottle cell formation**

To confirm that bottle cell induction by known regulators was reproducible in my hands, I tested the activities of Nodal, the related ligand Activin, and the downstream transcription factor Smad2 in ectopic induction of apical constriction. Overexpression of each was accomplished by injection of mRNA into the animal pole at the 2-cell stage, and ectopic apical constriction was assessed by checking for dark spots of accumulated pigmentation at or around stage 10, a standard assay for ectopic apical constriction (Kurth and Hausen, 2000). I have found that, because pigmentation is not always uniform even within single unmanipulated embryos, that it is important to observe pigmentation accumulation over time as a truer metric of apical constriction. The original assay for ectopic apical constriction also tracked pigment over time (Kurth and Hausen, 2000), although more recent reports have forgone this level of detail (Choi and Sokol, 2009; Ossipova et al., 2015b).

As has been published by other groups, injection of each mRNA individually was sufficient to induce ectopic apical constriction (Figure 2.1). Activin was a particularly strong inducer, with the lowest dose tested (15 pg, not shown) being sufficient. In many cases, *activin* and *xnr1* induced ectopic apical constriction strongly enough to cause invagination of the entire animal hemisphere. The resulting extreme tension reversed or prevented endogenous bottle cell formation, as seen in the vegetal view (Figure 2.1). The inducing activities of *xnr2* and *smad2* were not as strong, but might cause similar results at higher doses. For comparison, *shrm3* mRNA was injected, and caused mild ectopic constriction and no defects in endogenous bottle cell formation.
Predicted Nodal targets are expressed in or near forming bottle cells

In work now published, the Julie Baker lab conducted ChIP-seq experiments using anti-Smad2/3 antibodies to identify direct transcriptional targets of Nodal signaling in hESCs (Kim et al., 2011). Early on in the analysis, a list of targets strongly regulated by Nodal was assembled. From this preliminary data, I selected candidates that exhibited the highest fold increase after Nodal signaling activation to pursue as potential regulators of bottle cell formation. These included *fzd8, lhx1, sel1l, epha4, ephb3, pcdh7, nxn, gadd45a*, and *has2*.

In the simplest case, direct inducers of bottle cell formation will be expressed in bottle cells themselves, or very nearby. To test whether any of the candidates were expressed in this manner, I performed in situ RNA hybridization using probes complementary to each gene’s transcript. Several candidates are expressed near the first forming bottle cells, in the dorsal marginal zone or more broadly (Figure 2.2). Those expressed only in the dorsal marginal zone have comparable expression patterns to the Spemann-Mangold organizer marker, *goosecoid*. Whether any of these candidates are expressed in bottle cells themselves is difficult to determine using this method, since in situ hybridization may not reveal low levels and because the accumulated pigmentation used to identify bottle cells may obscure some staining. Quantitative methods such as qPCR are challenging to do for bottle cells, since the population is so small; to assess expression of these genes in more abundant ectopically-induced apically constricting cells may not reflect endogenous expression (see Discussion).

Expression in the marginal zone is not particularly surprising for targets of Nodal signaling, but is consistent with a role in bottle cell induction. Dorsal enrichment of these targets is also to be expected, since Nodal signaling is most active on the dorsal side. It should be emphasized that dorsal target gene expression is near the very first bottle cells to form, again consistent with a role in induction. However, not all of these candidates are expressed throughout the marginal zone, i.e., near where bottle cells will form at later stages. Expression of these genes at later stages, as bottle cells continue to form, was not tested here. In any case, although consistent with a role in bottle cell induction, the expression patterns alone cannot demonstrate such a role for these candidates, dorsally or otherwise.

Overexpression of predicted Nodal targets is not sufficient to induce ectopic apical constriction

A gain-of-function approach was used to test whether these candidates may indeed have bottle cell inducing activity. Using *activin* overexpression as a positive control, each candidate was overexpressed by microinjection and assessed for ability to induce ectopic apical constriction in the animal hemisphere as indicated by pigment accumulation.

As shown in Figure 2.3, no candidate alone was sufficient to induce ectopic apical constriction. Unlike the *activin* positive control, in which animal hemisphere cells apically constrict so much as to nearly be internalized, no pigment accumulation beyond natural variation in coloration was observed upon Nodal target overexpression. Furthermore, endogenous bottle cells formed as usual in
each case. The exception, as usual, was the activin control; in this case, embryos experienced so much contraction of the animal hemisphere that apical constriction to form bottle cells was physically impossible.

**Overexpression of Wnt5a, Vangl2, or Lgl is not sufficient to induce ectopic apical constriction**

I next turned to three other published bottle cell inducers, Wnt5a, Vangl2, and Lgl (Choi and Sokol, 2009; Ossipova et al., 2015b). Using a similar approach, I overexpressed each by mRNA injection. However, in contrast to the expected results obtained using Nodal etc., at no dose did I find that any of the three was sufficient to induce ectopic apical constriction (Figure 2.4 and data not shown). Consistent with this, endogenous bottle cell formation was not impaired. Even using high doses of wnt5a mRNA made from the same plasmids used in the publication, generously gifted by the authors, ectopic apical constriction was never induced (Figure 2.5a). In a few cases, injected embryos had very mild accumulations of pigment; however, this was never more than is observed naturally in unmanipulated embryos. Even the strongest accumulations looked much more similar to the cell death seen after the physical wounding inherent to injections, and very unlike true apical constriction. Finally, none of the pigmentation spots got darker over time, unlike apical constriction induced by Nodal signaling factors.

The negative results for Wnt5a were particularly puzzling, especially because Wnt5a-overexpressing embryos did have the convergent extension defects typical of embryos with disrupted PCP signaling (Figure 2.5b). In other words, the injected mRNA was having the predicted effects on the morphogenetic movement most famously associated with Wnt5a function, but no effects on bottle cell formation. This phenotype confirms the injected mRNA was functional at later stages, ruling out technical problems, e.g., failure to synthesize stable mRNA or poor injection technique.

Even overexpression of a receptor along with Wnt5a did not induce ectopic apical constriction or affect endogenous bottle cell formation. Fz7, a receptor that participates in Wnt/PCP signaling (Djiane et al., 2000; Kraft et al., 2012; Kim et al., 2008) and canonical signaling through β-catenin (Zhang et al., 2013), and Fz8, also implicated in both (Deardorff et al., 1998; Wallingford et al., 2001), failed to facilitate induction of ectopic apical constriction by Wnt5a (Figure 2.6). As for Wnt5a alone, co-injected embryos formed normal endogenous bottle cells, and did display convergent extension defects later in development (not shown).

**Wnt/PCP pathway members are expressed near forming bottle cells**

Wnt5a has been thoroughly characterized as a ligand that activates Wnt/Planar Cell Polarity (Wnt/PCP) signaling (Moon et al., 1993; Wallingford et al., 2001; Oishi et al., 2003; Schambony and Wedlich, 2007; Qian et al., 2007; Cha et al., 2008) as well as canonical signaling through β-catenin in the *Xenopus* embryo (He et al., 1997; Mikel and Nusse, 2006). Although I was unable to reproduce data showing that Wnt5a itself can induce bottle cells, it remains possible that PCP signaling components are involved. Indeed, in the years since my work on this project, data supporting a role for Vangl2 and Daam1 has been published (Ossipova
et al., 2015b). As for the Nodal targets, I tested whether members of the Wnt/PCP pathway are expressed in a manner consistent with a potential role on bottle cell induction. RNA *in situ* hybridization patterns for selected pathway members shows expression in the marginal zone, particularly on the dorsal side (Figure 2.7). This has been published by other groups, and is appropriate for PCP signaling’s known role in convergent extension of dorsal tissues at later stages (reviewed in Keller, 2002; Roszko et al., 2009; Vladar et al., 2009; Wallingford et al., 2002; Singh and Mlodzik, 2012; reviewed in Wu and Mlodzik, 2009).

Like the Nodal targets discussed above, PCP genes are not necessarily expressed in forming bottle cells themselves. Although these components are transmembrane or cytosolic proteins, not secreted ligands like Wnt5a, it is possible that they may still act at a distance. This would not be unusual for PCP signaling, which is known to have long-range effects in some cases (Classen et al., 2005; Vinson and Adler, 1987; Wu and Mlodzik, 2009).

In addition, not all members of the pathway might be required in this case. Perhaps Wnt5a is engaging in a non-planar polarity mode of signaling, using interaction partners normally associated with PCP. It is important to distinguish between PCP signaling *components* in this context from *planar cell polarity* itself, especially since bottle cells have not been shown to exhibit planar polarity to date. PCP components have been shown to be involved in non-planar polarity establishment or maintenance in other cases (Dollar et al., 2005; Ossipova et al., 2015a; Cha et al., 2011; Tao et al., 2009). What other Wnt-activated signaling mechanism PCP components might execute here remains unknown.

**Overexpression of Wnt/PCP pathway members is not sufficient to induce ectopic apical constriction**

Given their expression patterns, consistent with but not demonstrative of a role in bottle cell induction, I manipulated PCP component expression to reveal potential functions. To test this, I first overexpressed members of the pathway and assessed whether they could induce ectopic apical constriction. Embryos were injected in the animal hemisphere with the indicated dose of mRNA (Figure 2.8a).

In no case was ectopic pigment accumulation observed. Uneven pigmentation was never beyond the normal variation seen in uninjected controls. However, overexpression of every PCP component tested did result in a shortened body axis (Figure 2.8b), similar to the convergent extension defects widely documented for PCP genes. These data confirm that overexpression of PCP genes was effective later in development. Thus, although these candidates had the expected roles in gastrulation movements, these results suggest that they are not sufficient to induce apical constriction.

Like my data concerning Wnt5a, the results of Vangl2 overexpression obtained here are in conflict with published work supporting a role in bottle cell induction (Ossipova et al., 2015b). Although mRNA concentration was estimated using a NanoDrop spectrophotometer, it remains possible that my work has not managed to use the precise dose that yielded ectopic apical constriction in the published works. Those publications do not indicate whether injections were
titrated to identify a particular functional dose, or whether the dose used caused a convergent extension phenotype.

**Knockdown of Wnt/PCP pathway member expression can inhibit Activin-induced ectopic bottle cell formation, but does not inhibit endogenous bottle cell formation**

My results are at odds with published data showing that PCP component overexpression is sufficient to induce ectopic apical constriction. Those papers also illustrated a requirement for the tested PCP components in ectopically induced apical constriction. I tested the requirement for the PCP components Vangl2, Celsr/Flamingo, and Inversin in ectopic Activin-induced apical constriction. As shown in Figure 2.9, apical constriction is robustly induced by *activin* mRNA injection. Importantly, co-injection of a morpholino targeting any of the three PCP components causes a reduction in Activin-induced apical constriction. This is in agreement with published data that PCP components knockdown can inhibit Activin- (Choi and Sokol, 2009) or Shroom3-induced (Ossipova et al., 2015b) ectopic apical constriction.

However, central to the conclusions drawn by those authors is also that these components are also necessary for *endogenous* bottle cell formation. I attempted to reproduce some of the published experiments, and to test for a requirement for other PCP components in bottle cell formation. Vegetal injection of the same morpholinos that decrease ectopic apical constriction failed to inhibit endogenous bottle cell formation (Figure 2.10). At the highest doses tested, bottle cell formation was delayed, but a normal blastopore ring always formed. There was also no observable defect observed in terms of the order or pattern of bottle cell formation; bottle cells formed dorsally first, then laterally, and finally ventrally, as in uninjected controls. PCP component knockdown was successful, as embryos did exhibit convergent extension defects later in development (not shown).

These results conflict with published data supporting a requirement for PCP signaling (or, at least, PCP components) in bottle cell formation. The published results show only a single time point for these experiments. It may be true that bottle cells also formed at a delay in those cases.

**Vegetal overexpression of TGF-β ligands shifts the blastopore ring towards the animal hemisphere**

One fascinating difference between endogenous bottle cell formation and ectopically induced apical constriction is that the former occurs only in a very spatially restricted manner, whereas the latter occurs en masse with no evident pattern or boundary. In addition, key regulators of other instances of apical constriction (e.g., Shrm3, a hingepoint cell regulator) are absent in bottle cells. Yet another difference is that PCP components appear to be required for ectopic apical constriction but not endogenous bottle cell formation, according to my work. As such, while ectopic apical constriction is a useful tool for characterizing the roles of potential bottle cell inducers, it is not a perfect representation of the endogenous situation.
To understand more about bottle cells themselves, and to begin to address why they form in such a restricted pattern, I overexpressed TGF-β ligands by vegetal mRNA injection and observed the consequences for bottle cells. Unlike animal overexpression, this manipulation does not induce massive apical constriction; instead, bottle cells form in as restricted a manner as in uninjected controls. However, bottle cells consistently formed further from the vegetal pole, such that the blastopore ring formed was larger and more marginal (Figure 2.11). Strikingly, the blastopore ring was always completed, although often with a delay; whether more bottle cells than usual formed to create this larger ring is unclear. Because the blastopore ring was larger, more yolk was contained within it. As a result, though the blastopore started to close, it never closed completely, even at later stages (not shown). A large yolk plug remained, and embryos failed to gastrulate and soon died. In summary, neither endogenous nor experimentally elevated levels of TGF-β signaling induce bottle cell formation beyond a spatially restricted ring, which is always complete despite blastopore size. This suggests that an inhibitor may prevent apical constriction from occurring more broadly, even in the presence of elevated TGF-β signaling.

**Neither activation nor inhibition of FGF signaling affects TGF-β induced ectopic apical constriction**

The animal pole-ward shift in bottle cell formation observed upon vegetal overexpression of TGF-β ligands is reminiscent of the shifts in mesodermal gene expression when \(fgf8\) is overexpressed (Fletcher, 2006). FGF signaling has been shown to play complex roles in bottle cell biology. For example, it promotes cell cycling, which must be arrested to allow bottle cell formation (Kurth, 2005). However, overexpression of a dominant negative FGF receptor can cause failures in blastopore formation and closure (Amaya et al., 1991; Amaya et al., 1993). To further characterize the role of FGF signaling in bottle cell formation, I used the ectopic apical constriction induction assay described above, and either activated or inhibited FGF signaling to characterize its effects on ectopic apical constriction. Previous publications have performed similar experiments, and demonstrated a role for FGF signaling in this context (Kurth and Hausen, 2000).

As usual, animal overexpression of TGF-β ligands caused ectopic apical constriction at varying degrees of severity (Figure 2.12). Coinjection of 500pg dominant negative FGFR2-encoding mRNA or \(fgf8\) mRNA had no appreciable affect on this phenotype beyond normal variation. In most cases, cojected embryos also failed to make or maintain endogenous bottle cells, likely due to the physical strains induced by drastic ectopic apical constriction. In conclusion, at the mRNA doses used, modulation of FGF signaling did not abrogate or accelerate Activin-induced ectopic apical constriction.
2.3 Discussion

In short, no novel regulators of bottle cell formation were identified. Consistent with published work, ectopic activation of Nodal-family TGF-β signaling was sufficient to induce ectopic bottle cells, as was overexpression of shroom3. Although some direct targets of Nodal signaling are expressed near endogenous bottle cells, none of those tested in overexpression experiments were sufficient to induce ectopic apical constriction. Likewise, members of the Wnt/Planar Cell Polarity pathway are expressed near bottle cells, but even at doses that cause characteristic convergent extension defects, none are sufficient to induce apical constriction ectopically. Furthermore, for the components tested in knockdown experiments, PCP members are not required for endogenous bottle cell formation. Some of these data, though reproducible in my hands, are in conflict with publications showing a role for PCP in this cell population.

Nodal signaling and targets

Nodal signaling has been well established as a pathway required and sufficient to induce bottle cell formation. Transcriptional activation of targets downstream of Nodal must be required, as interfering with Smad2/3 or its cofactors leads to a failure in bottle cell formation (Wills and Baker, 2015; Schier, 2003; Shen, 2007), and both endogenous and ectopic bottle cell formation do not occur until after zygotic genome activation. Nodal signaling is also required for mesendoderm induction during the same developmental stages. How this signaling pathway regulates the patterning events of germ layer induction and the morphogenetic events of gastrulation remains a major question. For bottle cells, specifically, future work may show whether or not the Nodal targets that regulate germ layer identity are the same as those that regulate apical constriction in this context.

Perhaps more than one Nodal target gene is required for bottle cell formation; this could explain why overexpression of individual targets was not sufficient to induce apical constriction ectopically. It is possible that overexpression of some combinations of these genes could be sufficient. Additionally, knockdown of these targets in presumptive bottle cells may yet reveal a role for them in the endogenous case. Furthermore, in the scope of this project, only a handful of Nodal targets was tested, compared to the number identified in the work referenced (Kim et al., 2011). As mentioned above, these candidates were selected from preliminary data showing a large fold change in expression upon activation of Nodal signaling. Any of the remaining targets, alone or in combination, may have a role in bottle cell formation that has yet to be identified.

Relatedly, the TGF-β ligands known to be sufficient for induction of ectopic apical constriction also simultaneousaly activate mesodermal and endodermal gene expression (Asashima et al., 1999; Kurth and Hausen, 2000). These germ layer patterning outputs of Nodal signaling may be required to create a permissive
environment for bottle cell induction. Direct inducers of bottle cells may only succeed in inducing apical constriction if a similar environment is already established. However, Wnt5a, Lgl, Vangl2, and Daam1 have been published to induce ectopic apical constriction (Choi and Sokol, 2009; Ossipova et al., 2015b); whether their activity in this context also influences mesendodermal gene expression was not tested. Although not discussed in detail here, endocytosis of apical membrane and cell cycle arrest are also required for bottle cells to form (Lee and Harland, 2010; Kurth, 2005). Regulators of these processes, Nodal targets or otherwise, may also be required to induce ectopic apical constriction.

Interestingly, both vegetal overexpression and animal overexpression of Nodal family ligands have similar effects on the visible location of bottle cell formation. In both cases, the blastopore appears to be shifted towards the animal pole. However, the bases for this shift are likely to be different in each case. Over-activation of Nodal signaling in the vegetal hemisphere induces ectopic expression of some mesodermal marker genes (Agius et al., 2000), such that the chemical environment where bottle cells normally form is altered by patterning events. This environment may be defined by the interface between endoderm and mesoderm, for example through some inhibitor of apical constriction whose expression is also shifted animaly with this interface. Animal overexpression, on the other hand, does not expand endogenous Nodal signaling or shift the endoderm-mesoderm boundary animaly. Instead, drastic ectopic apical constriction in the animal cap, combined with apparent physical constraints within the embryo, mechanically prohibit or reverse endogenous bottle cell apical constriction. Thus, also both manipulations shift the blastopore and can cause subsequent defects in gastrulation, the basis in each case is different. Relevant differences might be described in more detail by measuring the distance of blastopore formation from the vegetal pole, either in units of distance or number of cell diameters.

The shift in either case, though, may shed light on the normally restricted nature of bottle cell formation. Endogenous formation might be restricted by patterned expression of a molecular inhibitor; alternatively, mechanical features of the embryo my impose a restriction on where bottle cells can form, such that only a few in a certain location can generate enough force to productively constrict. However, the shift in bottle cells shows that they can form at different longitudinal positions in the embryo, and furthermore, that they still always form in a narrow band or ring. Bottle cells also form in a normal pattern in tissue explants, such as Keller sandwiches (Keller and Danilchik, 1988). Their ability to form in potentially different mechanical environments suggests that it is a molecular mechanism that restricts their formation. This remains a hypothetical argument in the context of my work, as I did not directly test any mechanical properties of tissues or cells in my experiments.

One appealing candidate for a molecule that restricts bottle cell formation is the Nodal transcriptional target and inhibitor Lefty. Lefty restricts the range of Nodal signaling during mesoderm induction and left/right patterning (Cheng et al.,
Notably, overexpression of *lefty* can inhibit endogenous bottle cell formation (Wills and Baker, 2015). Furthermore, knockdown of *lefty* increases the likelihood of exogastrulation; although the blastopore forms completely, it does not close (Branford and Yost, 2002). This is reminiscent of the phenotype observed after vegetal overexpression of Nodal or Activin (Figure 1.11), and close inspection suggests that ectopic apical constriction may be occurring in the animal hemisphere of lefty morphants. Continued investigation may show a role for Lefty or other Nodal inhibitors in bottle cell formation.

**Wnt/PCP component signaling**

Despite using published reagents at published concentrations and reproducing published convergent extension phenotypes, no manipulations of Wnt/PCP signaling I performed support a role for this pathway in endogenous bottle cell formation. Several controls, discussed below, could be performed to confirm these findings. Consistent with previous work, however, knockdown of PCP components was able to inhibit ectopically induced apical constriction. My data suggest that overexpression of PCP components is not sufficient to induce ectopic apical constriction. In comparison with convergent extension as a PCP-regulated movement, this may not be surprising. Overexpression of PCP components does not induce ectopic convergent extension, and in fact interferes with normal convergent extension movements (Moon et al., 1993; Adler et al., 2000; Tree et al., 2002; Bastock, 2003). Phenotypes of these embryos look similar to those caused by PCP component knockdown. This is likely caused by an imbalance of particular PCP proteins polarized to opposing sides of the apical membrane. Like convergent extension, then, apical constriction may require precise levels of all PCP components, such that simple overexpression of single components cannot induce this cell shape change.

One result in agreement with the literature (Choi and Sokol, 2009) is that Wnt11, which, like Wnt5a, is required for convergent extension, does not induce ectopic apical constriction. This suggests that not every mechanism of Wnt/PCP activation can induce apical constriction. Perhaps Wnt5a and other PCP-associated molecules (Vangl2, Daam1) are acting through a non-planar cell polarity pathway to regulate apical constriction.

Knockdown experiments summarized in Figure 2.9 confirm that knockdown of the PCP components Celsr/Flamingo, Inversin, and Strabismus inhibits Activin-induced ectopic constriction. However, based on other experiments using the same morpholinos, my data suggest that the PCP components tested are not required for endogenous bottle cell formation (Figure 2.10). At high doses, bottle cells form after a delay; previous reports do not show multiple time points, stating only that bottle
cells failed to form. Perhaps extended observations in those experiments would show bottle cell formation after a delay, as I have shown. The finding, reproduced here, that PCP component knockdown partially inhibits ectopically induced apical constriction is interesting, but may not reflect a role in endogenous bottle cells.

Several controls could be employed to confirm that Wnt/PCP is being successfully manipulated, but not causing a bottle cell phenotype. Western blotting for PCP proteins after mRNA injection could be performed to demonstrate successful overexpression; recent advances in imaging polarized PCP components in *Xenopus* could also show whether components are localized aberrantly *in vivo* (Butler and Wallingford, 2015). Higher resolution observations of convergent extension movements could also lend support for the known functions of PCP components. A wider range of doses, either of mRNA or morpholino, could be injected to manipulate expression levels more strongly. CRISPR/Cas9 technology could also be used to make PCP mutants (Doudna and Charpentier, 2014) and assess the consequences for endogenous bottle cell formation. Analysis of apical constricting cells that contribute to gastrulation in other species may also shed light on the role of PCP in these populations.

It is not intuitively clear how a pathway that regulates planar cell polarity might regulate apical constriction, a process that requires establishment of apicobasal polarity but not planar polarity. Shrinkage of the apical membrane does not occur isometrically in bottle cells (Hardin and Keller, 1988), but this is likely due to physical constraints imposed by the embryo as a whole, rather than cell polarity. Recent work has identified a role for certain PCP signaling components in regulating apicobasal polarity of bottle cells and other cell types (Marsden and DeSimone, 2001; Dollar et al., 2005; Ossipova et al., 2015a; Cha et al., 2011; Tao et al., 2009), but whether these components are acting through mechanisms associated with PCP signaling is unclear. It is worth noting that some PCP components have been shown to be required for hingepoint cell formation by apical constriction during neural tube closure (Williams et al., 2014; Nishimura et al., 2012; McGreevy et al., 2015). Whether this pathway is required for other instances of apical constriction remains to be seen.

**Additional regulation**

Other features of bottle cell formation, not addressed by the experiments described here, merit consideration. Particularly fascinating is the progressive, but not strictly sequential, formation of bottle cells from the dorsal side early on to the lateral and later ventral sides of the blastopore. It is remarkable that the blastopore forms as a contiguous ring of bottle cells, despite these cells often forming out of order and not initially directly adjacent to one another. This may also reflect aspects of the mechanism of induction: for example, since bottle cells do not form strictly in order, that does not lend support for an inducing signal which is passed directly from bottle cell to bottle cell. A simpler explanation could invoke a pair of
molecules distributed such that they form a boundary orthogonal to the animal-vegetal axis, roughly at the interface between endodermal and mesodermal germ layers. Nodal and its target and inhibitor Lefty fit this description, and have both been shown to have a role in bottle cell formation (Agius et al., 2000; Wills and Baker, 2015).

To expand on that subject, it is worth considering manipulations that induce ectopic blastopore rings. For example, ventral activation of Wnt signaling by mRNA injection induces a secondary axis, the first external indication of which is a secondary blastopore lip on the ventral side. Furthermore, this ectopic blastopore lip meets up to connect with the endogenous lip as they each proceed laterally, and the embryo undergoes gastrulation relatively normally (personal observation, Funayama et al., 1995; Guger and Gumbiner, 1995). Global activation of canonical Wnt signaling by treatment with lithium chloride generates a blastopore in which all bottle cells form at nearly the same time, with no directional progression from one site to another (personal observations not shown; Fredieu et al., 1997; Kao and Elinson, 1988). Embryos ventralized by UV irradiation exhibit a similar behavior (Jansen et al., 2007). Again, a simple explanation is that they are restricted to a particularly longitudinal position by some molecular interaction along the animal-vegetal axis.

The role of Wnt/β-catenin signaling in bottle cell formation bears discussion. Bottle cells form first on the dorsal side, a region of high Wnt activity. However, this pathway is not likely to be a direct inducer of bottle cells. First, due in part to canonical Wnt signaling activity (Agius et al., 2000), the dorsal side also experiences higher Nodal signaling than elsewhere in the embryo, and Nodal is already known to be required for bottle cell formation. Second, bottle cells form on the lateral and ventral sides later, far from cleavage stage Wnt signaling. As discussed above, a signal (here, perhaps Wnt) that initiates bottle cell formation on the dorsal side could in theory be propagated by a bucket-brigade system, but this is not necessarily consistent with the order of bottle cell formation. Third, although ectopic activation of Wnt signaling on the ventral side does induce a secondary blastopore lip, ectopic activation in the animal cap does not induce apical constriction. Finally, and perhaps most importantly, bottle cells and a blastopore form normally when Wnt signaling has been inhibited in the embryo (Glinka et al., 1998; Kazanskaya et al., 2000; Kiecker and Niehrs, 2001; Jansen et al., 2007).

Intact microtubules and endocytosis-driven membrane remodeling are also required for bottle cell formation (Lee and Harland, 2007; Lee and Harland, 2010). Dynamin and Rab5 are required in this population, and endocytic machinery has also been implicated in other cases of apical constriction, including hingepoint cell formation (Lee and Harland, 2010; Ossipova et al., 2014; Ossipova et al., 2015b). Endeavors to identify direct regulators of endocytosis and/or microtubules will likely complement efforts to characterize regulators of actomyosin in apically constricting cells, including bottle cells.
My experiments took the approach of testing candidate Nodal targets and signaling molecules to try to identify direct bottle inducers. An aspect of bottle cell formation that my work has not touched on directly is the cytoskeletal and junctional structures involved. One way to identify additional bottle cell regulators could be to take a “bottom up” approach, and test known regulators of these structures and associated cell biological processes. For example, regulators of myosin activity such as ROCK, Rho, other or regulators of actin polymerization such as Arp2/3, formins, or the Rho GTPases Rac, Rho, and Cdc42 have been shown to play various roles in apically constricting cells (reviewed in Sawyer et al., 2010; Martin and Goldstein, 2014; Munjal and Lecuit, 2014). Likewise, testing the contribution of junctional proteins including cadherins, catenins, vinculin, etc. and their regulators will continue to reveal their roles.

Ideally, what is known about the signaling molecules and pathways that regulate bottle cell formation will be connected with downstream cell polarity and cytoskeletal regulators to generate a complete picture of how apical constriction is regulated in the case of bottle cell formation. The candidate approach has succeeded in identifying a few players, but by nature is limited to molecules already characterized to some degree. Techniques such as RNA- or ChIP-sequencing or mass spectrometry might hold an appeal in this case, since they can identify new candidates in an unbiased manner. Unfortunately, certain inherent features of bottle cells pose challenges to these unbiased approaches. First, they constitute a tiny population in comparison to the rest of the embryo, and there are no known unique molecule markers that could be used to sort them. Second, bottle cells are defined by their shape change, and therefore impossible to isolate before that shape change occurs, by which time key regulators may no longer be expressed or even required. Third, although much larger numbers of apical constricting cells can be induced ectopically by known regulators of endogenous bottle cells, it is clear that these ectopic cells are not equivalent in every way to bottle cells, and may not accurately represent the endogenous situation. Thus, although an analysis of ectopic apical constriction would likely identify new regulators of this shape change in general, it would not necessarily lead to the discovery of bottle cell-specific regulatory molecules or mechanisms. Mutagenesis screens, for example through the use of CRISPR/Cas9 technology, might prove useful in identifying regulators in the future.

It is of interest to characterize multiple different kinds of apical constriction, both because they are fascinating on their own and because misregulation of apical constriction can cause serious defects during development and in homeostasis (reviewed in Sawyer et al., 2010; Martin and Goldstein, 2014). Failure of bottle cell formation in particular can lead to exogastrulation; failure of apical constriction in analogous cells in other species can cause a range of gastrulation defects. Later developmental events also require apical constriction, and failures here can lead to birth defects in humans; for example, neural tube closure defects due to failed hingepoint cell apical constriction can cause spina bifida. Because apical constriction is closely associated with many instances of epithelial-to-mesenchymal
transition (EMT), this broadens the scope of how knowledge about regulatory mechanisms might be applied. In particular, apical constriction and EMT can contribute to tumor metastasis. A deep comprehension of the many mechanisms that can regulate apical constriction will be necessary to understand and potentially treat these associated disease states.

Final thoughts

Despite the scarcity of data identifying direct inducers of endogenous bottle cells, some salient features serve as clues to what regulators might be. First, it seems almost inevitable that a transcriptional target of Nodal will be involved. Not only is Nodal one of the few signaling ligands required, but overexpression of Nodal pathway transcriptional activators (i.e., Smad2/3) is also sufficient to induce apical constriction. Furthermore, both endogenous bottle cell formation and ectopic apical constriction induced by Nodal pathway activation do not occur until after the mid-blastula transition, suggesting that each requires zygotic gene expression. Second, despite this apparent requirement, non-transcriptional events may also be necessary. These may involve regulation of apicobasal or even planar cell polarity by Wnt/PCP components, as well as other intracellular factors, such as Lgl. These players may also directly influence the cytoskeleton or intracellular trafficking events to drive apical constriction. Finally, besides the timing of their appearance, the location and order in which bottle cells form cannot be ignored. The input of canonical Wnt signaling, whether directly or through elevation of Nodal signaling or other mechanisms, induces the earliest bottle cells in both endogenous and ectopic scenarios. Most fascinating might be the tightly restricted and non-sequential pattern of bottle cell formation, which has yet to be recapitulated in any ectopic context. The activity of an inhibitor of apical constriction could account in part for this phenomenon. Thus, in addition to bottle cell inducers downstream of Nodal and other signaling, it will be important to identify inhibitors as well, to accurately characterize the endogenous regulation of this instance of apical constriction.

A deeper understanding of bottle cell biology will contribute to a more nuanced view of gastrulation in relation to germ layer induction. Both subjects have been thoroughly investigated over the years, and developmental biologists have long recognized that their spatial and temporal coordination is likely important for normal development. Through molecular mechanisms we are continuing to characterize, patterning and morphogenesis during this stage are exquisitely orchestrated such that, just as the three germ layers have been established, morphogenetic movements initiate to arrange them spatially for the functions of their respective derivatives in the embryo. Bottle cell induction in relation to mesendoderm induction are a part of this remarkable feat, and characterization of how they are coordinated remains an exciting challenge in the field.
2.4 Methods

Collection, microinjection, and culturing of *Xenopus laevis* embryos

Adult *Xenopus laevis* females were injected between 4:00 and 6:00pm with 500 units of human chorionic gonadotropin (Merck Animal Health) and housed at 16°C overnight. The following morning, eggs were manually squeezed from gravid females and fertilized *in vitro* using mashed pieces of testis in Modified Barth’s Saline (MBS) from a male euthanized no more than two weeks prior. Fertilized eggs were kept in 1/3 Modified Frog Ringer’s (1/3 MR) at 12°C, 16°C, or room temperature for at least half an hour before being dejellied in 3% cysteine (Sigma-Aldrich) for 10 minutes, followed by five rinses in 1/3MR.

Needles for microinjection were pulled on a Model P-87 Flaming/Brown micropipette puller (Sutter Instrument Company) using the following program setting: Heat=820, Pull= 50, Vel.=140, Time=44. Microinjections were performed under a Stemi SV 6 stereomicroscope (Carl Zeiss Microscopy, LLC.) using a Picospritzer II (General Valve Corporation) and microinjection manipulator (Narishige Group).

Embryos were microinjected into one cell at the 2-cell or the 4-cell stage. For each blastomere injected, the following doses were used, always in either 10nL: morpholinos (GeneTools), 20ng total; mRNAs, 500pg each unless otherwise noted. Embryos were injected in 2.5% Ficoll PM400 (GE Healthcare) in 1/3MR, and allowed to heal for at least 30 minutes before being transferred into 1/3MR. Embryos were cultured at 12°C, 16°C, or room temperature until they reached the desired stage, at which point they were collected for analysis. Embryos were staged according to Nieuwkoop and Faber, 1994.

Preparation of mRNA and morpholinos for injection

mRNA was synthesized from linearized template DNA using the Sp6 or T7 mMessage mMachine Transcription Kits (Ambion, Life Technologies) according to manufacturer’s instructions. mRNA was cleaned up by a wash in 70% ethanol, a wash in isopropanol + 0.5M ammonium acetate, and a final wash in 70% ethanol. mRNA concentration was quantified using a NanoDrop ND-1000 Spectrophotometer, and aliquots were stored at -80°C at up to 500ng/µL.

Morpholinos were resuspended in RNase/DNase-free water at 1mM or 2mM and stored in the dark at room temperature, except for the fluoresceinated standard morpholino, which was kept at -20°C.

RNA *in situ* hybridization

Embryos were fixed in 4% formaldehyde, 0.1M MOPS, pH 7.4, 2 mM EGTA, 1 mM MgSO4 (MEMFA) for 2-4 hours at room temperature or overnight at 4°C; they then underwent one 95% ethanol wash before being washed and stored in methanol at -20°C. RNA *in situ* hybridization was performed using digoxygenin (DIG)-labeled probes, anti-DIG-AP Fab fragments (Roche), and BM Purple (Roche) as published (Harland, 1991).
Figure 2.1 Ectopic TGF-β signaling is sufficient to induce ectopic apical constriction

Embryos were injected in the animal hemisphere with the indicated doses of mRNA into 1 blastomere at the 2-cell stage, and imaged 24-26 hours later at stages 9, 10, and 11.5. Accumulation of pigment was used as a proxy for apical constriction, as published (Kurth and Hausen, 2000). Representative embryos are shown, with quantification in the lower right-hand corner of each 2hr time point panel.
Figure 2.2  Targets of Nodal signaling are expressed near forming bottle cells

RNA in situ hybridization using probes complementary to direct Nodal signaling transcriptional targets shows their expression in the marginal zone. Most are enriched in the dorsal marginal zone, near the first forming bottle cells, visible as a dark line of pigmentation at the dorsal blastopore lip. Representative embryos are shown, with quantification in the lower right-hand corner of each panel. The expression pattern of the organizer gene goosecoid (gsc) is included for comparison. Vegetal view; dorsal side up.
Figure 2.3 Nodal signaling targets do not induce ectopic apical constriction

Embryos were injected in the animal hemisphere with the indicated doses of mRNA into 1 blastomere at the 2-cell stage, and imaged 24-26 hours later at stages 9, 10, and 11.5. Accumulation of pigment was used as a proxy for apical constriction, as published (Kurth and Hausen, 2000). Representative embryos are shown, with quantification in the lower right-hand corner of each 2hr time point panel. The activin condition was included as a positive control.
Figure 2.4 Overexpression of *wnt5a*, *stbm*, or *lgl* is not sufficient to induce ectopic apical constriction

Embryos were injected in the animal hemisphere with the indicated doses of mRNA into 1 blastomere at the 2-cell stage, and imaged 24-26 hours later at stages 9, 10, and 11.5. Accumulation of pigment was used as a proxy for apical constriction, as published (Kurth and Hausen, 2000). Representative embryos are shown, with quantification in the lower right-hand corner of each 2hr time point panel.
Figure 2.5a Different doses of *wnt5a* do not induce ectopic apical constriction

Embryos were injected in the animal hemisphere with the indicated doses of mRNA into 1 blastomere at the 2-cell stage, and imaged 24-26 hours later at stages 9, 10, and 11.5. Accumulation of pigment was used as a proxy for apical constriction, as published (Kurth and Hausen, 2000). Representative embryos are shown, with quantification in the lower right-hand corner of each 2hr time point panel.
Figure 2.5b  Overexpression of \textit{wnt5a} causes characteristic and dose-dependent defects in convergent extension/axial elongation

Embryos from the cohort shown in Figure 2.5a were imaged after 48 and 72 hours to assess the effects of \textit{wnt5a} injection at later stages. Representative embryos are shown. Lateral view; anterior to the left.
Figure 2.6 Co-injection of frizzled receptors does not potentiate activity of wnt5a to induce ectopic apical constriction

Embryos were injected in the animal hemisphere with the indicated doses of mRNA into 1 blastomere at the 2-cell stage, and imaged 24-26 hours later at stages 9, 10, and 11.5. Accumulation of pigment was used as a proxy for apical constriction, as published (Kurth and Hausen, 2000). Representative embryos are shown, with quantification in the lower right-hand corner of each 2hr time point panel.
**Figure 2.7 Components of the Wnt/PCP pathway and apicopasal polarity genes are expressed near forming bottle cells**

RNA *in situ* hybridization using probes complementary to PCP pathway and apicobasal polarity genes shows their expression in the marginal zone. Most are enriched in the dorsal marginal zone, near the first forming bottle cells, visible as a dark line of pigmentation at the dorsal blastopore lip. Representative embryos are shown, with quantification in the lower right-hand corner of each panel. The expression pattern of the organizer gene *goosecoid (gsc)* is included for comparison. Vegetal view; dorsal side up.
Figure 2.8a Overexpression of Wnt/PCP components is not sufficient to induce ectopic apical constriction

Embryos were injected in the animal hemisphere with the indicated doses of mRNA into 1 blastomere at the 2-cell stage, and imaged 24-26 hours later at stages 9, 10, and 11.5. Accumulation of pigment was used as a proxy for apical constriction, as published (Kurth and Hausen, 2000). Representative embryos are shown, with quantification in the lower right-hand corner of each 2hr time point panel. The activin condition is included as a control; stbm and wnt5a are included to demonstrate that their failure to induce apical constriction is reproducible.
Figure 2.8b  Overexpression of Wnt/PCP genes causes expected defects in convergent extension/axial elongation

Embryos from the cohort shown in Figure 2.8a were imaged after 48 and 72 hours to assess the effects of wnt5a injection at later stages. Representative embryos are shown. For pk1a and wnt5a, two phenotypes were observed: shortening of the anterior-posterior axis, and defects in gastrulation and neural tube closure. For these conditions, an embryo representative of each phenotype is shown. Lateral view; anterior to the left.
Figure 2.9 Knockdown of PCP components partially inhibits Activin-induced ectopic apical constriction

Embryos were injected with the indicated doses of mRNA and morpholino into 1 blastomere at the 2-cell stage, and imaged 24-26 hours later at stages 9, 10, and 11.5. Accumulation of pigment was used as a proxy for apical constriction, as published (Kurth and Hausen, 2000). Representative embryos are shown, with quantification in the lower right-hand corner of each 2hr time point panel.
Figure 2.10 Knockdown of PCP components delays, but does not prevent, endogenous bottle cell formation

Embryos were injected on the vegetal side with the indicated doses of morpholino in 1 blastomere at the 2-cell stage, and imaged 24-27 hours later, starting at stage 9.
Representative embryos are shown, with quantification in the lower right-hand corner of each final time point panel. Vegetal view.
Figure 2.11 Vegetal overexpression of TGF-β ligands shifts the blastoporal ring animally

Embryos were injected on the vegetal side with the indicated doses of mRNA in 1 blastomere at the 2-cell stage, and imaged 25-26 hours later, starting at stage 10. Representative embryos are shown, with quantification in the lower right-hand corner of each final time point panel. Vegetal view.
Figure 2.12 Activation or inhibition of FGF signaling does not inhibit ectopic apical constriction induced by TGF-β ligands

Embryos were injected on the animal side with the indicated doses of mRNA in 1 blastomere at the 2-cell stage, and imaged 24-26 hours later, starting at stage 9. Representative embryos are shown, with quantification in the lower right-hand corner of each final time point panel.
Chapter 3: Characterizing the roles of \textit{sall1} and \textit{sall4} in neural patterning and morphogenesis

3.1 Introduction

Another critical shaping event in the vertebrate embryo is that of neurulation, during which the exterior, flat neural plate is converted in a hollow neural tube just under the dorsal surface of the embryo (Smith and Schoenwolf, 1997; Schoenwolf, 1991; reviewed in Wallingford, 2005). This dorsal neural tube goes on to form the brain and spinal cord of the larval and adult organism. Two major movements contribute to neural tube closure: hingepoint cell formation and convergent extension. Hingepoint cell formation, as mentioned in Chapter 1, is another instance of apical constricting: one medial and two dorsolateral (one on the left side of the embryo, one on the right) populations of neural plate cells undergo apical constriction to generate kinks in the neural plate, along which the plate folds (Schoenwolf and Franks, 1984; Lawson et al., 2001; Colas and Schoenwolf, 2001). Convergent extension serves to elongate the anteroposterior axis of the whole embryo (Keller et al., 1992; Keller et al., 2008a; Schoenwolf and Alvarez, 1989), thereby narrowing the dorsal ectodermal tissue of the neural plate and facilitating its folding and fusing along the midline to form a tube.

Molecular regulation of both hingepoint cell formation and convergent extension has been characterized to an extent. The actin-binding protein Shroom3 is required for hingepoint cell formation and sufficient to induce ectopic apical constriction (Hildebrand and Soriano, 1999; Haigo et al., 2003). Shroom3 has been shown to act with Rho kinase downstream of the FERM domain protein Lulu, Trio and other RhoGEFs, p120-catenin, and RhoA to recruit an active actomyosin network to the apical side (Hildebrand, 2005; Nakajima and Tanoue, 2012; Plageman et al., 2011; Lang et al., 2014; Nishimura and Takeichi, 2008). Adhesion and trafficking proteins are also required for hingepoint cell formation (Nandadasa et al., 2009; Ossipova et al., 2014; Badouel et al., 2015), as are other cytoskeletal regulators, including Abl, Arg, Vinculin, and Mena (Koleske et al., 1998; Xu et al., 1998; Lanier et al., 1999). Convergent extension is driven by Wnt/Planar Cell Polarity (Wnt/PCP) signaling (Sokol, 1996; Tada and Smith, 2000; Wallingford et al., 2000; Darken et al., 2002; Wallingford and Harland, 2002; Moon et al., 1993; Ohkawara, 2003; Oishi et al., 2003). Interestingly, PCP components have also been shown to be required for hingepoint cell formation (Nishimura et al., 2012; Williams et al., 2014; McGreevy et al., 2015), shedding new light on the importance of this pathway in neural tube closure. Many other genes are associated with neurulation or neural tube closure defects (Copp et al., 2003; Wallingford et al., 2013; Harris and Juriloff, 2010), but their mechanistic roles have yet to be described.

Like gastrulation, the morphogenetic movements of neurulation happen in close temporal proximity to patterning events in the same tissue (see Figure 1.2). Neural induction and patterning has been described by the activation-transformation model proposed by Nieuwkoop (Nieuwkoop, 1952a; Nieuwkoop,
During gastrulation, antagonism of BMP signaling on the dorsal side induces neural identity in the dorsal ectoderm (Wilson and Hemmati-Brivanlou, 1995; Suzuki et al., 1997; Schmidt et al., 1995; Sasai et al., 1994; Sasai et al., 1995; Hawley et al., 1995; Smith and Harland, 1992; Hemmati-Brivanlou et al., 1994; Khokha et al., 2005; reviewed in Stern, 2005), and (Ozair et al., 2013). Different fates along the anteroposterior axis are then specified in the resulting neural plate by the activity of the FGF, retinoic acid, and Wnt signaling pathways (Durston et al., 1989; Cox and Hemmati-Brivanlou, 1995; Niehrs, 1999; Maden, 2006; reviewed in Ozair et al., 2013). In the caudal neural plate, Wnt signaling acts through a canonical β-catenin-dependent pathway to induce posterior fates (e.g., spinal cord). Rostrally, where Wnt is not active due to the activity of antagonists, anterior fates (e.g., forebrain) are adopted (McGrew et al., 1995; Bouwmeester et al., 1996; Glinka et al., 1998; Domingos et al., 2001; Kiecker and Niehrs, 2001). The role of the Wnt/β-catenin pathway in neural posteriorization has been well documented for decades, but the mechanisms by which this pathway acts had remained uncharacterized until recently.

Previous work used a variety of techniques to identify putative direct Wnt/β-catenin transcriptional targets in the posterior neural plate (Young et al., 2014). Our goal was to investigate these targets to determine which mediate Wnt signaling’s posteriorizing effects in the neural plate. Among the list of many targets were the transcription factor-encoding genes sall1 and sall4. These genes piqued our interest because of their responsiveness to and dependence on Wnt signaling, endogenous expression in the frog neural plate, and known activity as transcriptional regulators in other contexts.

sall1 and sall4 are two vertebrate orthologs of the Drosophila spalt (sal) gene, which encodes a transcriptional regulator best known for its roles in wing imaginal disc patterning and specification of the embryonic termini (de Celis and Barrio, 2009; Jürgens, 1988; Kühnlein et al., 1994). These transcription factors can function as either activators or repressors (Zhang et al., 2006; Lim et al., 2008; Lauberth et al., 2007; Yang et al., 2012). Of the vertebrate homologs, sall1-4, sall1 and sall4 are implicated in kidney, limb, heart, and nervous system development (Buck et al., 2001; Baringa Ender and Bronner-Fraser, 2004; Neff et al., 2005; Nishinakamura et al., 2001; Onuma et al., 1999; Kiefer et al., 2003; Camp et al., 2003). In humans, mutations in sall1 cause Townes-Brocks syndrome (Kohlhase et al., 1998); symptoms include polydactyly, hearing loss, and kidney and heart defects (Powell and Michaelis, 1999). Mutations in sall4 are associated with Okihiro Syndrome (Kohlhase et al., 2002), patients of which display eye, limb, heart, and kidney defects (Kohlhase et al., 2003). Both sall1 and sall4 regulate pluripotency in some contexts: for example, sall1 contributes to oct4 activation in embryonic stem cells (Zhang et al., 2006), and sall4 is required for proliferation of the Inner Cell Mass (ICM) in mice, for proliferation of embryonic stem cells (ESCs) in culture, and for efficient induction of induced pluripotent stem cells (iPSCs) (Sakaki-Yumoto et al., 2006; Wu et al., 2006; Elling et al., 2006; Tsubooka et al., 2009).
We previously showed that \textit{sall4} is required for posterior neural patterning in \textit{Xenopus}, as would be expected for a gene with a function downstream of Wnt in this process (Young et al., 2014). Knockdown of \textit{sall4} causes a posterior shift in and/or reduction of posterior neural marker gene expression; later, neural differentiation is also impaired. Further, members of the \textit{pou5f3} family of genes are upregulated in \textit{sall4}-deficient tissue. This family consists of three genes: \textit{pou25} (now known as \textit{pou5f3.2}; www.xenbase.org), \textit{pou60} (\textit{pou5f3.3}), and \textit{pou91} (\textit{pou5f3.1}). These are the \textit{Xenopus} homologs of mammalian \textit{oct3/4}, which are well known for their role of maintaining pluripotency, for example during nuclear reprogramming as well as in naturally-occurring stem cells (Rosner et al., 1990; Schöler et al., 1998; Niwa et al., 2000; Niwa et al., 2002; Takahashi and Yamanaka, 2006; Kim et al., 2009; reviewed in Jerabek et al., 2014; Radzisheuskaya and Silva, 2014). The \textit{pou5f3} genes also maintain an undifferentiated state during early \textit{Xenopus} development by inhibiting maternal and zygotic signaling (Cao et al., 2004; Cao et al., 2006; Cao et al., 2007; Morrison and Brickman, 2006; Livigni and Brickman, 2013), and regulate neural differentiation at later stages (Snir et al., 2006; Archer et al., 2011). Importantly, concurrent knockdown of the \textit{pou5f3} family with knockdown of \textit{sall4} is sufficient to partially rescue posterior neural marker gene expression, demonstrating that the patterning defects in \textit{sall4} morphants are due at least in part to derepression of \textit{pou5f3} family genes. Though originally identified as a Wnt target in this tissue, \textit{sall4} also acts downstream of other signaling inputs into neural patterning, namely FGF and retinoic acid signaling: knockdown of \textit{sall4} prevents posteriorization by overexpression of \textit{fgf8} or treatment with all-trans retinoic acid (Young et al., 2014).

Along with the patterning defects associated with \textit{sall4} knockdown, we noticed accompanying morphogenetic defects, in that neural tube closure partially or completely failed in \textit{sall4} morphant tissue. A role for \textit{sall} genes in neural morphogenesis in mouse has been documented (Böhm et al., 2008). One goal of my thesis work was to investigate this previously unexplored aspect of \textit{sall4} function during \textit{Xenopus} neural development, and to understand the cellular basis for this gross morphogenetic defect.

Although \textit{sall1} was identified as a Wnt target in the same study that characterized \textit{sall4}, the roles of \textit{sall1} in \textit{Xenopus} neural development were left comparatively unexplored. While \textit{sall1} and \textit{sall4} might regulate overlapping sets of target genes, it is reasonable to expect that they each may regulate some unique genes as well. The four Sall proteins differ in sequence and in structure; one striking difference between Sall1 and Sall4 is that the latter lacks the fourth of five Zinc finger domains (Sweetman and Münsterberg, 2006). Furthermore, the \textit{sall} genes differ in their expression patterns and regulate different developmental processes (Camp et al., 2003; Farrell and Münsterberg, 2000; Kohlhase et al., 1998; Kohlhase et al., 2002; reviewed in de Celis and Barrio, 2009; Sweetman and Münsterberg, 2006). Thus, the contribution of \textit{sall1} to neural development in \textit{Xenopus} remained an open question.
The main focus of my thesis work was to further characterize the roles of *sall1* and *sall4* in neural patterning, morphogenesis, and differentiation. As discussed below, both genes are required for these aspects of neural development, likely because of their endogenous function as repressors of the *pou5f3* family of pluripotency genes. I pursued this hypothesis by working to characterize the influence of the *pou5f3* family on neural development.

### 3.2 Results

**sall1 and sall4 are expressed in the neural plate throughout neurulation**

As previously shown (Young et al., 2014), *sall1* and *sall4* are expressed in the neural plate throughout neurulation (Figure 3.1). At early neurula stages, they are also expressed in the non-neural ectoderm (Figure 3.1A,E). Their expression becomes more restricted as development proceeds. Posterior expression of *sall1* is maintained throughout neurulation, and anterior domains begin to express *sall1* in a more restricted fashion later (Figure 3.1B-D). Similarly, *sall4* is also expressed posteriorly throughout stages 13-19, but acquires striking brain expression domains during mid-neurula stages (Figure 3.1F-H). These expression patterns are consistent with a role downstream of Wnt signaling in the posterior neural plate, but also suggest possible roles in anterior neural tissue, especially during later development.

**sall1 and sall4 are each required for anteroposterior neural patterning**

To confirm the requirement for *sall4* in neural patterning, and to investigate whether *sall1* has a similar role, I knocked down the function of each gene and assayed for different neural fates along the anteroposterior axis. Knockdown was performed in only the right half of the embryo, such that the morphant half could be compared to the unmanipulated left half as well as to wholly unmanipulated embryos. Two non-overlapping morpholinos were used for each experiment (see Appendix I); these blocked either splicing or translation, and had complementarity to either *sall1* RNA or *sall4* RNA, but not both. Neural fates were assayed using RNA *in situ* hybridization with probes against known markers of regional neural fates. Results are summarized in Figure 3.2 and Figure 3.7b.

In no case is neural identity lost, as shown by approximately normal expression of the pan-neural marker *sox2* (Figure 3.2M-P'). As had already been shown, however (Young et al., 2014), *sall4* is required for posterior neural fate induction. Without its function, spinal cord (*hoxB9*) and hindbrain (*krox20*) markers are reduced or shifted posteriorly, indicating a loss of posterior identity and a slight expansion of anterior identity (compare Figure 3.1C,C' to Figure 3.1A). The forebrain marker *otx2* is not strongly affected. Other markers of the neural plate and neural border (*pax6* and *pax3*) are also lost in the posterior, while anterior expression is largely unaffected (compare Figure 3.1G,G',K,K' to Figure 3.1E,I). Similar losses are seen when *sall1* function is knocked down, suggesting that these two *sall* genes have a similar role (compare Figure 3.1B,B',F,F',J,J' to Figure 3.1A,E,I).
Critically, though, their functions are not entirely redundant, because knockdown of each individually is sufficient to cause a posterior loss phenotype. In addition, knocking them both down at once does not cause more drastic defects (Figure 3.1D,D',H,H',L,L'). These data show a newly identified function of *sall1* and demonstrate that both *sall1* and *sall4* are required for anteroposterior patterning of the neural plate.

Knockdown of *sall1* or *sall4* appears to have a cell- or tissue-autonomous effect on these neural marker gene expression patterns, as marker gene expression on the uninjected left side was unaffected in all cases.

**sall1 and sall4 are each required for neural differentiation**

The consequences of disrupted neural patterning in *sall* morphants persist as development proceeds. Neuronal differentiation is impaired in the absence of either *sall1* or *sall4*, as shown in Figure 3.3A-D by decreased expression of *tubb2b*. Occasionally, although expression of *tubb2b* itself is reduced, the expression domain appears to be shifted laterally; however, this does not show an actual shift or expansion of the domain, and is instead an effect of the morphogenetic defects exhibited by these embryos: see below. Defects in differentiation persist to later stages (Figure 3.3E-H).

Interestingly, differentiation is impaired in the anterior as well as in the posterior. This differs from the patterning defects in *sall* morphants, where anterior marker gene expression is largely unperturbed. However, the differentiation phenotype is not inconsistent with the expression patterns of *sall1* and *sall4*; as seen in Figure 3.1, they are expressed throughout the neural plate at early and, to a lesser extent, later stages.

**sall1 and sall4 are each required for neural tube closure**

As mentioned above, we noticed during previous work that *sall4* knockdown appeared to cause neural tube closure defects, but had not described or investigated these morphogenetic problems in detail. To characterize the roles of *sall1* and *sall4* in neural tube morphogenesis, I performed knockdown experiments similar to those described above, and documented the consequences for neural tube closure. Results are summarized in Figure 3.4 and Figure 3.9.

In contrast to control embryos, *sall1* and *sall4* morphant embryos indeed fail to close their neural tubes. Although the unmanipulated left side forms a normal neural fold that bends towards the midline, the morphant side remains flat, failing to form an elevated neural fold (compare Figure 3.4B-D,B'-D' to Figure 3.4A). Bilaterally injected morphants fail to elevate a neural fold on both sides (Figure 3.4F-I). Neural tube closure defects persist, and later in development the neural tissue can be seen exposed on the surface of the embryo (Figure 3.4J-M, unilateral morphants; bilateral morphants not shown). Interestingly, embryos can still twitch and swim (not shown), suggesting that failed neural tube close does not prevent neuronal differentiation and function on the uninjected side; this is consistent with the normal expression of *tubb2b*, as seen above.

It should be emphasized that, as with patterning and differentiation, morphogenesis of the uninjected side of manipulated embryos is not perturbed.
This again supports a tissue-autonomous role for the \textit{sall} genes. More explicitly, these data show that there is neither a molecular nor mechanical dependence of one half of the embryo on the other for correct neural patterning, morphogenesis, or differentiation in this context.

\textit{sall1} and \textit{sall4} are each required for hingepoint cell formation

To determine the underlying cause of the gross morphogenetic defects of \textit{sall} morphant embryos, I used confocal microscopy to investigate the cellular behaviors of neural plate cells known to be important for neural tube closure. In particular, I assessed cytoskeletal features and cell shape of the hingepoint cells, which normally undergo apical constriction to generate the kinks that elevate the neural folds and bend them towards the midline (Schoenwolf and Franks, 1984; Lawson et al., 2001; Colas and Schoenwolf, 2001). Results comparing \textit{sall} morphants to control embryos are summarized in Figure 3.5 and quantified in Figure 3.10.

Actin accumulation in the apical domain of neural plate cells was clearly visible in control tissue, but noticeably reduced in \textit{sall1} and/or \textit{sall4} morphant tissue (Figure 3.5, green; compare Figure 3.5B-D,E,F to Figure 3.5A; higher magnification views shown in A'-F'). In addition, morphant cells failed to undergo apical constriction or associated apicobasal elongation, as visualized by antibody staining for the microtubule protein DM1 (Figure 3.5, red). Upon closer inspection of cell morphologies, morphant cells often appeared rounded up, without even the cuboidal morphology of control non-hingepoint cells.

The absence of hingepoint cells is accompanied by a failure of neural plate bending. Morphant tissue stays flat, consistent with the gross phenotype of splaying neural plate. In other words, the failure in cell shape change co-occurs with the predicted consequent tissue shape change. Thus, it is likely that failed hingepoint cell formation is an underlying cause of failed neural tube closure in \textit{sall1} and \textit{sall4} morphants.

\textit{pou5f3} family genes are derepressed in the absence of \textit{sall1} and/or \textit{sall4}

One published role of \textit{sall1} and \textit{sall4} is the regulation of pluripotency. In some cases, \textit{sall} family genes have been shown to regulate the transcription of the stem cell factors, including \textit{oct4} (Sakaki-Yumoto et al., 2006; Wu et al., 2006; Zhang et al., 2006). To test whether this was true during \textit{Xenopus} neural development, \textit{sall1} and \textit{sall4} were knocked down, and morphant embryos were stained via RNA \textit{in situ} hybridization for expression of \textit{pou5f3} family genes, the \textit{Xenopus} homologs of \textit{oct4}.

Expression of all three family members (\textit{pou25}, Figure 3.6A-D; \textit{pou60}, Figure 3.6E-H; and \textit{pou91}, Figure 3.6I-L) is increased in \textit{sall1} and \textit{sall4} morphant tissue Figure 3.6. This increase is confirmed by qPCR (Figure 3.6M-O). These results support a role for \textit{sall1} and \textit{sall4} in transcriptional repression of \textit{pou5f3} family gene expression in the neural plate.

Interestingly, as seen in the qPCR data, it seems possible that \textit{sall1} may play a stronger role in regulation of \textit{pou25} than does \textit{sall4} (Figure 3.6M), and conversely, that \textit{sall4} may more strongly repress \textit{pou60} (Figure 3.6N). These differences in function may provide an instance of distinct functions of \textit{sall1} and \textit{sall4}.
**pou5f3 gene overexpression disrupts anteroposterior neural patterning**

Because the *pou5f3* genes, homologs of the stem cell maintenance factor *oct4*, are upregulated in tissue lacking *sall1* and/or *sall4* function, we hypothesized that this was what could be preventing neural tissue from differentiating. In other words, elevation of *pou5f3* expression could be sufficient to account for the several defects observed in *sall* morphants. To test this, we overexpressed the *pou5f3* genes individually in neural tissue to determine whether this was sufficient to cause the same defects, without experimental manipulation of *sall1* or *sall4*.

Overexpression of any individual member of the *pou5f3* family is sufficient to cause a reduction and/or shift in posterior neural marker gene expression (Figure 3.7a, compare panels B-D,F-H,J-L to panels A,E,I; Figure 3.7b). Expression of *sox2* is only mildly affected (Figure 3.7a, compare panels N-P to panel M). This phenotype is strikingly similar to that observed in *sall* morphants (see Figure 3.2). These data suggest that the defects in posterior induction observed in *sall* morphants could be explained by *pou5f3* gene derepression.

**pou5f3 gene overexpression prevents neural differentiation**

To test for a role in maintaining pluripotency and preventing differentiation of neural tissue, I overexpressed *pou5f3* genes and assayed for neural differentiation using the marker *tubb2b*. Consistent with their well-known role as pluripotency factors, overexpression is sufficient to prevent differentiation and expression of this neuronal marker (Figure 3.8, compare panels B-D to panel A). Again, this phenotype is similar to that of *sall* morphants. It should be noted that overexpression of each individual *pou5f3* gene alone is sufficient to cause this defect; indeed, at least at the dose used (250pg), differentiation appears to be impaired even more than in *sall* morphants.

**pou5f3 gene overexpression prevents neural tube closure**

Using a similar approach, I addressed whether *pou5f3* overexpression is also sufficient to cause the morphogenetic defects associated with *sall* knockdown. Again, overexpression of any individual *pou5f3* family member is sufficient to prevent neural tube closure (Figure 3.9). The neural plate on the manipulated side fails to fold, and instead remains flat (Figure 3.9 compare panels B-D to panel A). As in *sall* morphants, this defect persists into later development (not shown). These data suggest that the neural tube closure defects of *sall* morphants could be due to *pou5f3* gene derepression.

Overexpression of the *pou5f3* genes can also cause gastrulation defects (not shown). These may contribute to the open neural tube phenotype, in cases where the blastopore does not close completely and the yolk plug is a physical obstruction to tube closure.

**pou5f3 gene overexpression prevents hingepoint cell formation**

Suspecting that the underlying cellular basis of neural tube closure defects might be the same in *pou5f3*-overexpressing and *sall* morphants embryos, I again performed confocal microscopy to examine cell shape. As in *sall* morphants, actin fails to accumulate in *pou5f3*-overexpressing neural tissue (Figure 3.10, compare
panels B-D, B’-D’ to panels A, A’). Furthermore, characteristically wedge-shaped hinge point cells also fail to form. As for \textit{sall} morphants, this cellular defect is likely to contribute to failed neural tube closure. These data suggest that the failure of hinge point cells to form in \textit{sall} morphants is due to the derepression of \textit{pou5f3} genes.

A quantification of phenotypes in \textit{sall1} and \textit{sall4} morphants and \textit{pou}-overexpressing embryos is shown in Figure 3.10. Four phenotypic classes are included: the most normal case for controls in a symmetrically closing tube with symmetrical actin signal on the left and right sides (blue bars), as shown in the representative images; the most common case for morphant and \textit{pou}-overexpressing embryos is to have one side splaying open and with very little actin signal compared to the internal control side (red bars), also shown in the representative images; more rarely, embryos have symmetrical actin signal but one splaying fold (orange bars) or a closing tube but symmetrical actin signal (green bars). Distribution of phenotypes is similar among controls and similar among \textit{sall} morphant and \textit{pou}-overexpressing conditions, and these two groups differ substantially from each other.

In summary, overexpression of \textit{pou5f3} genes is sufficient to cause the same defects observed in \textit{sall1} and/or \textit{sall4} morphant tissue. It is thus likely that \textit{pou5f3} derepression in \textit{sall} morphants contributes to the morphant phenotype, including defects in patterning, morphogenesis, and differentiation, and furthermore may be sufficient to account for those phenotypes.

### 3.3 Discussion

Known functions of the \textit{sall} and \textit{pou5f3} gene families

The \textit{sall} gene family has been shown to play diverse roles in metazoan development, including touch-sensitive neuron specification in \textit{C. elegans} (Mitani et al., 1993); wing imaginal disc patterning, photoreceptor specification, and trachea formation in \textit{Drosophila} (de Celis and Barrio, 2000); (Mollereau et al., 2001; Domingos et al., 2004; Kühnlein and Schuh, 1996; Franch-Marro and Casanova, 2002); and limb development and regeneration, kidney development, and neural crest migration in vertebrates (King et al., 2003; Neff et al., 2005; Neff et al., 2011; Koshiba-Takeuchi et al., 2006; Camp et al., 2003; Nishinakamura et al., 2001; Barembaum and Bronner-Fraser, 2004). This family is also associated with a variety of human disease states, including Townes-Brocks Syndrome and congenital kidney defects associated with \textit{sall1} mutations (Kohlhase et al., 1998; Botzenhart et al., 2007; Jain et al., 2007; Liapis, 2004) and Okihiro Syndrome and some types of leukemia associated with \textit{sall4} (Al-Baradie et al., 2002; Kohlhase et al., 2002; Ma et al., 2006; Cui et al., 2006). This work describes a new role for \textit{sall1} in neural patterning, morphogenesis, and differentiation in the frog \textit{Xenopus laevis}, and expands on the role of \textit{sall4} during neural development in this species to include a
requirement in morphogenesis.

In *Xenopus*, *sall1* and *sall4* are expressed in limbs during normal development (Neff et al., 2005), and the pattern of *sall4* expression in regenerating limbs is suggestive of a role in promoting dedifferentiation (King et al., 2003; Neff et al., 2011). Both genes are expressed in embryonic stem cells (Sakaki-Yumoto et al., 2006), and have been found to inhibit differentiation in various contexts (Karantzali et al., 2011; Wu et al., 2006; Elling et al., 2006; Zhang et al., 2006; Tsubooka et al., 2009). In these cases, *sall1* and *sall4* function to maintain an undifferentiated state, often by interacting with and/or positively regulating pluripotency factors such as Nanog and Oct3/4. In stark contrast, their key role in the frog neural plate is to repress the pluripotency factors of the *pou5f3* family to allow differentiation. These opposite functionalities of *sall1* and *sall4* may occur through the use of different *cis* regulatory elements or cofactors. Contextual factors such as these might account for their activities as both transcriptional activators and repressors (Zhang et al., 2006; Lim et al., 2008; Lauberth et al., 2007; Yang et al., 2012). Further analysis of the transcriptional targets of the *sall* family could shed light on the dual nature of their function. RNA-sequencing on *sall1* and *sall4* morphants, as described below under “Preliminary experiments and future directions,” is underway to identify putative targets. ChIP-seq using antibodies against endogenous *sall1* and *sall4* or against tagged versions (e.g., FLAG-tagged constructs) would provide more direct evidence of transcriptional regulation. This could be compared to existing datasets mapping open or closed chromatin states or binding of transcriptional activators or repressors (Akkers et al., 2009; van Heeringen et al., 2014; Wills et al., 2014) throughout the *Xenopus* genome to further characterize the regulation of potential *sall1* and *sall4* targets. Upcoming improvements to the *X. laevis* genome will facilitate this analysis (see http://www.xenbase.org/).

The *sall* genes themselves are regulated by a variety of inputs in different contexts. Wnt, FGF, Shh, EGFR, and BMP signaling have all been shown to impinge on their expression (reviewed in de Celis and Barrio, 2009; Sweetman and Münsterberg, 2006). FGF and Shh regulate *sall3* in the *Medaka* hindbrain (Koster et al., 1997; Carl and Wittbrodt, 1999); Wnt, FGF, and BMP regulate *sall1* in the chicken limb (Carl and Wittbrodt, 1999; Farrell and Münsterberg, 2000). Our lab has shown that both *sall1* and *sall4* are targets of Wnt signaling, and that at least *sall4* also acts downstream of FGF and retinoic acid signaling in neural development (Young et al., 2014). Interestingly, *sall* gene function can either enhance Wnt signaling (Sato et al., 2004) or attenuate it (Onai et al., 2004). Cellular context, including binding partners or other cofactors, likely modifies both *sall* regulation and function.

The *pou5f3* family has been shown to repress differentiation in *Xenopus* (Cao et al., 2004; Cao et al., 2006; Snir et al., 2006; Cao et al., 2007; Archer et al., 2011). Additionally, simultaneous knockdown of all family members causes defects in axial elongation and in both posterior and anterior neural patterning (Morrison and Brickman, 2006). Recently, a list of putative Oct4/PouV targets conserved between *Xenopus*, mouse, and human has been identified; this list is enriched for regulators
of epithelial development and cell adhesion (Livigni and Brickman, 2013). These results may explain why embryos depleted of both sall1 or sall4 and pou5f3 family function develop so poorly (e.g. failed gastrulation, tissue integrity defects; see “Preliminary experiments and future directions” below). Temporal control over sall and pou5f3 function, for example through the use of inducible constitutively active or repressive fusions that would allow normal development until the state in question, could allow finer dissection of the function of these genes.

Downstream of sall1 and sall4 in the Xenopus neural plate – effects on morphogenesis

Failure of hinge point cell formation is evident based on staining for F-actin and for microtubules. Apices do not constrict in morphant tissue, and cells to not elongate or take on a wedge morphology. However, it is unclear how these cytoskeletal and cell shape changes are regulated by sall1 and sall4, two transcription factors. Several regulators are known to be required for this instance of apical constriction, including Shroom3, RhoGEFs, RhoA, Rho kinase, and active myosin (Hildebrand, 2005; Nakajima and Tanoue, 2012; Plageman et al., 2011; Lang et al., 2014; Nishimura and Takeichi, 2008). The key regulator Shrm3 is of particular interest, given its central role in recruiting the apical actomyosin network, which is clearly disrupted in sall morphants. I was unable to detect Shrm3 or phosphorylated myosin by immunofluorescence, perhaps because many available antibodies do not recognize the Xenopus epitopes. Overexpression of a GFP-tagged Shroom3 causes gastrulation defects that preclude analysis at neural stages (personal observation, not shown). Thus, visualization of key apical constriction machinery has remained elusive. Tertiary amplification strategies could be used to detect very low doses of a tagged Shroom3 to avoid gastrulation defects. Alternatively, an antibody could be raised to detect endogenous Xenopus laevis Shroom3.

Perhaps these known regulators of apical constriction are transcriptional targets of the sall genes, such that their expression is lost in sall morphants. The sall genes may additionally or alternatively regulate other, as yet unknown regulators of hinge point cell formation. RNA sequencing of sall morphants may give some clues about the connections between sall gene function and hinge point cell formation (see below). It should be noted that although the sall1 and sall4 expression patterns are much broader than the regions in which hinge point cell form, the derepression of pou5f3 family genes through the neural plate could account for the failure of this restricted population to form.

It remains unclear how apical constriction driving hinge point cell formation is restricted to three discrete stripes from anterior to posterior in the Xenopus neural plate. In the mouse, medial hinge point cell formation is induced by an unidentified signal from the underlying notochord, whereas the dorsolateral hinge points are inhibited by Shh and BMP signaling and positively regulated by
Noggin (Ybot-Gonzalez et al., 2002; Ybot-Gonzalez et al., 2007). The roles of these molecules in *Xenopus* hingepoint cell formation have not yet been investigated. The *sall1* and *sall4* morphant phenotypes do not shed light on this issue, beyond suggesting a general requirement for these transcription factors in neural differentiation. It seems unlikely that *sall1* and *sall4* regulate hingepoint cells directly, and more likely that apical constriction is one of many developmental events that cannot occur in *sall*-morphant tissue.

It should be noted that the discussion of neural morphogenesis here is limited to primary neurulation, the rolling of a flat sheet into a tube. This mode of neurulation occurs in the rostral portion of vertebrate embryos (Smith and Schoenwolf, 1997; Colas and Schoenwolf, 2001). Secondary neurulation, which occurs via formation of a solid column and subsequent epithelialization to form a hollow tube, occurs in the caudal portion of these embryos (Cirley, 1969; Gont et al., 1993; Catala et al., 1995; Griffith, 1997; reviewed in Lowery and Sive, 2004). The role of *sall1* and *sall4* in secondary neurulation remains unaddressed by my work. However, as the neural tube of *sall1* and/or *sall4* morphants remains open to both the anterior and posterior extents of the *Xenopus* embryo (Figure 3.4), it is possible that these genes play a role in both types of neurulation in these embryos.

Not discussed at length here, *sall* morphants exhibit shortened anteroposterior axes suggestive of convergent extension defects. These likely contribute to neural tube closure defects, although they may not affect patterning. Convergent extension movements are regulated by the Wnt/Planar Cell Polarity (Wnt/PCP) pathway (Sokol, 1996; Tada and Smith, 2000; Wallingford et al., 2000; Habas et al., 2001a; Darken et al., 2002; Wallingford and Harland, 2002; Moon et al., 1993; Ohkawara, 2003; Oishi et al., 2003), as introduced in Chapter 1 and discussed in Chapter 2. RNA in situ hybridization did not show an obvious change in expression of Wnt/PCP components in *sall* morphant tissue (not shown), but qPCR experiments to examine this in more detail have been unsuccessful for reasons not yet resolved. Beyond expression levels or domains, changes in the intracellular localization of PCP components can also disrupt pathway function (Strutt, 2001; Axelrod, 2001; Shimada et al., 2001; reviewed in Axelrod and McNeill, 2002; Roszko et al., 2009; Gray et al., 2011; Vladar et al., 2009; Wallingford, 2012); perhaps component localization, instead of or as well as expression, is regulated downstream of *sall1* and/or *sall4* such that convergent extension movement are disrupted upon *sall* knockdown. Wnt/PCP signaling has also been shown to have a role in hingepoint cell formation (Nishimura et al., 2012; Williams et al., 2014; McGreevy et al., 2015), so misregulation in *sall* morphants may contribute to neural tube closure defects through this route as well. Recently, visualization of asymmetrically localized Wnt/PCP proteins has been made possible in *Xenopus* (Butler and Wallingford, 2015); these techniques could be employed in *sall1* and *sall4* morphants to further assess PCP defects. More classical methods, such as generation of Keller sandwiches and measurement of their convergence and extension in culture (Keller and Danilchik, 1988), could also be used in conjunction with cell tracking software to quantify the defects axial elongation observed in *sall1*
This work has focused on the role of \textit{sall1} and \textit{sall4} in the neural plate. As shown in Figure 3.1, both genes are expressed beyond the neural plate, in the presumptive neural crest territory and epidermis. Earlier in development, the \textit{sall} genes are also expressed in the mesoderm (not shown). These genes may be required for development of these tissues; for example, what appear to be somite organization problems can be observed in some cases (Figure 3.5C,D). Ongoing work is beginning to address a role in non-neural tissue, for example by testing for expression of neural crest and paraxial mesoderm markers \textit{by in situ} hybridization in morphants. As the \textit{sall} family is associated with non-neural developmental processes and disease states, including limb development in \textit{Xenopus} and kidney, limb, and heart defects in Townes-Brocks Syndrome and Okihiro Syndrome (Neff et al., 2005; Kohlhase et al., 1998; Kohlhase et al., 2002; reviewed in de Celis and Barrio, 2009; Sweetman and Münsterberg, 2006), it is reasonable to expect that they will have functions in other tissues at this stage.

\textbf{Preliminary experiments and future directions}

Rescue experiments have proven very difficult for this work. Coinjection of \textit{sall1} or \textit{sall4} RNA does not rescue the posterior patterning phenotypes of \textit{sall} morphants (John Young, personal communication); additionally, overexpression of \textit{sall1} or \textit{sall4} by RNA injection alone does not cause a phenotype, at least in the assays used here. Furthermore, although it has been published that simultaneous knockdown of all \textit{pou5f3} family genes can rescue the \textit{sall4} morphant phenotype, coinjection of \textit{pou5f3} and \textit{sall1} or \textit{sall4} morpholinos (even at doses at which each morpholino is tolerated when injected alone) causes severe gastrulation defects, so neural development cannot be faithfully addressed.

Although the morphant phenotypes described here are true for two non-overlapping morpholinos each (one blocking splicing, the other translation) for both \textit{sall1} and \textit{sall4}, analysis of mutants will further validate the analyses presented here. Guide RNAs have been designed using CRISPRscan (Moreno-Mateos et al., 2015) against the \textit{Xenopus tropicalis} \textit{sall1} and \textit{sall4} genes to direct Cas9 mutagenesis in this related species. Bilateral knockdown will be performed in the future, with assessment of mutational efficiency to follow using the Surveyor kit assay (Integrated DNA Technologies) (Qiu et al., 2004). The assays described in this chapter will be used to assess patterning, morphogenesis, and differentiation in these mutants to determine whether their phenotype is similar to the \textit{Xenopus laevis} morphant phenotype. Encouragingly, preliminary experiments in \textit{X. tropicalis} show the expected open neural tube phenotypes when morphants are generated using \textit{tropicalis}-targeting morpholinos (both splice-blocking and translation-blocking).

RNA-sequencing on \textit{sall1} and \textit{sall4} morphant \textit{Xenopus laevis} embryos is also underway. Libraries have been generated, checked for quality, and submitted for
100 base pair paired-end sequencing. The resulting data sets will be analyzed for differential gene expression (see Methods below). Candidate targets will be validated through \textit{in situ} RNA hybridization and qPCR on \textit{sall1} and/or \textit{sall4} morphants. This analysis will reveal genes beyond the \textit{pou5f3} family that are regulated by \textit{sall1} and \textit{sall4}, some of which may regulate spinal cord gene expression or hingepoint cell biology.

An important caveat of this approach is that it might not identify obvious candidate regulators of apical constriction itself. As a hypothetical example, a gene with known roles in apical constriction may be passed over if the fold change in its expression is below an arbitrarily selected threshold. As another, even a gene that does show a fold change greater than that threshold may not be selected as a high-priority candidate if it has not yet been shown to have a role in apical constriction. As an additional limitation, for technical and practical reasons, RNAseq was performed on whole morphant embryos (embryos injected in the 2 dorsal blastomeres at the 4-cell stage, targeting primarily but not exclusively neural ectoderm); effects of this manipulation on cells outside the neural tissue could obscure some neural plate-specific changes. Nevertheless, this approach will be a significant step forward in characterizing the roles of \textit{sall1} and \textit{sall4} in development at this stage.

Final thoughts

Neural patterning and neural tube morphogenesis are coordinated in space and time to generate a functional central nervous system. Decades of research have characterized regulators of patterning, e.g., Wnt/\(\beta\)-catenin, FGF, and retinoic acid signaling, as well as regulators of morphogenesis, e.g., Wnt/PCP signaling, Shroom3. Both processes must occur together for proper development of this tissue, but the molecules and mechanisms known to regulate both patterning and morphogenesis together have only begun to be characterized. The work summarized here has identified new players and regulatory interactions in the coordination of neural development: \textit{sall1} and \textit{sall4} act downstream of patterning inputs to regulate differentiation via transcriptional repression of the \textit{pou5f3} family, thereby allowing both patterning and morphogenetic events to occur. This newly described role in \textit{Xenopus} neural development expands on the known roles of these conserved genes. Furthermore, though the mechanisms and targets through which \textit{sall1} and \textit{sall4} act have yet to be investigated in depth, their characterization in this context constitutes a significant step forward in our understanding of vertebrate neural development as a complete and coordinated system.
3.4 Methods

Collection, microinjection, and culturing of *Xenopus laevis* embryos

Adult *Xenopus laevis* females were injected between 4:00 and 6:00 pm with 500 units of human chorionic gonadotropin (Merck Animal Health) and housed at 16°C overnight. The following morning, eggs were manually squeezed from gravid females and fertilized *in vitro* using mashed pieces of testis in Modified Barth’s Saline (MBS) from a male euthanized no more than two weeks prior. Fertilized eggs were kept in 1/3 Modified Frog Ringer’s (1/3 MR) at 12°C, 16°C, or room temperature for at least half an hour before being dejellied in 3% cysteine (Sigma-Aldrich) for 10 minutes, followed by five rinses in 1/3MR.

Embryos were microinjected at the 2-cell stage (in one or both blastomeres) or the 4-cell stage (into the animal, dorsal, right blastomere, as identified by pigmentation) (Klein, 1987). Embryos were staged according to Nieuwkoop and Faber 1994. Successfully and correctly injected embryos were selected for analysis based on the presence of a coinjected lineage tracer (fluoresceinated control morpholino, *mem-gfp* mRNA, or *mem-rfp* mRNA; see below).

Needles for microinjection were pulled on a Model P-87 Flaming/Brown micropipette puller (Sutter Instrument Company) using the following program setting: Heat=820, Pull=50, Vel.=140, Time=44. Microinjections were performed under a Stevi SV 6 stereomicroscope (Carl Zeiss Microscopy, LLC.) using a Picospritzer II (General Valve Corporation) and microinjection manipulator (Narishige Group).

For each blastomere injected, the following doses were used, always in either 5nL or 10nL: morpholinos (GeneTools), 20ng total *sall1* and/or *sall4* morpholino plus 2ng standard fluoresceinated control morpholino (GeneTools); the micro-RNA targeting morpholino used as a control in Figure 3.6 has been shown not to have an effect on development (Song et al., 2014), and was injected at 20ng. mRNAs, including *mem-gfp* or *rfp* for lineage tracing, 500pg each unless otherwise noted. Embryos were injected in 2.5% Ficoll PM400 (GE Healthcare) in 1/3MR, and allowed to heal for at least 30 minutes before being transferred into 1/3MR + 50ng/mL gentamicin. Embryos were cultured at 12°C, 16°C, or room temperature until they reached the desired stage, at which point they were collected in one of several ways: see below.

Preparation of mRNA and morpholinos for injection

mRNA was synthesized from linearized template DNA using the Sp6 or T7 mMessage mMachine Transcription Kits (Ambion, Life Technologies) according to manufacturer’s instructions. mRNA was cleaned up by a wash in 70% ethanol, a wash in isopropanol + 0.5M ammonium acetate, and a final wash in 70% ethanol. mRNA concentration was quantified using a NanoDrop ND-1000 Spectrophotometer, and aliquots were stored at -80°C at up to 500ng/µL.

Morpholinos were resuspended in RNase/DNase-free water at 1mM or 2mM and stored in the dark at room temperature, except for the fluoresceinated standard morpholino, which was kept at -20°C.
RNA *in situ* hybridization

Embryos were fixed in 4% formaldehyde, 0.1M MOPS, pH7.4, 2 mM EGTA, 1 mM MgSO4 (MEMFA) for 2-4 hours at room temperature or overnight at 4°C; they then underwent one 95% ethanol wash before being washed and stored in methanol at -20°C. RNA *in situ* hybridization was performed using digoxygenin (DIG)-labeled probes, anti-DIG-AP Fab fragments (Roche), and BM Purple (Roche) as published (Harland, 1991).

Embryo sectioning and (immuno)fluorescent staining

Embryos were fixed in 4% paraformaldehyde + 0.25% glutaraldehyde in 10mM Na2HPO4, 1.8mM KH2PO4, 137mM NaCl, 2.7mM KCl (phosphate buffer saline, PBS) for 2-4 hours at room temperature or overnight at 4°C; they then underwent 3-5 PBS washes before being stored in PBS at 4°C. Embryos were mounted in 4% agarose and sectioned on a Pelco 101 Vibratome Series 1000 Sectioning System (Ted Pella, Inc.) using razor blades. Sections of either 100μm or 150μm were taken, and stored in PBS at 4°C.

Sections were washed twice with PBS + 0.1% Tween-20 (PBS-Tw), washed once for 10 minutes with PBS-Tw + 2mg/mL bovine serum albumin (BSA, Albumin from bovine serum, Sigma), and blocked in PBS-Tw + BSA + 10% goat serum (Gibco, Thermo Scientific) for at least 1 hour. Actin was stained with phalloidin-AlexaFluor488 (Molecular Probes, Invitrogen); microtubules were stained using mouse anti-DM1α primary antibody (Sigma Aldrich) and goat anti-mouse IgG secondary antibody conjugated to AlexaFluor555 (Life Technologies); DNA was labeled with 4’-6-diamino-2-phenylindole,dihydrochloride (DAPI, Molecular Probes, Invitrogen). All of these reagents were stored and used according to vendor instructions.

Confocal microscopy

Stained sections were mounted in PBS within silicone chambers on 3”x1”x1mm VWR VistaVision slides (VWR International, LLC.) under 22x30, 1.5 thickness Fisherbrand coverslips (Fisher Scientific). Mounted sections were imaged on a Zeiss LSM700 laser scanning microscope using the 20x objective. The Zen software system (Carl Zeiss Microscopy, LLC.) was used during image acquisition. Laser power was set to between 2 and 20. Digital Gain was set to between 400 and 750. The "best compromise" setting was used for acquisition, as there was little predicted cross-talk between channels. The FIJI image processing package (Abràmoff et al., 2004; Schneider et al., 2012) was used to digitally brighten and make maximum intensity Z-projections of confocal data.

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Individual embryos were homogenized in Trizol (Ambion, Life Technologies) using 21-gauge needles and 1 ml syringes, and homogenates were stored at -80°C. Total RNA was isolated using the TRizol Reagent according to manufacturer’s instructions (Ambion, Life Technologies). cDNA libraries were generated using the iScript Reverse Transcription Supermix or RT-qPCR (Bio-Rad) according to manufacturer’s instructions; no RT and no template controls were included with
each reaction. qPCR experiments were run using SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad) on a CFX96 Real-Time System, C1000 Touch Thermocycler (Bio-Rad). Statistical analysis was performed using Excel and Prism. Technical triplicates were averaged, and expression levels calculated relative to a reference gene (eef1a1) in the uninjected control using the 2^{-ΔΔCT} (Livak) Method (Livak and Schmittgen, 2001). Fold change across biological quadruplicates was normalized to the uninjected control (set by definition to a fold change of 1) and analyzed using ANOVA and Tukey’s honestly significant difference test. Asterisks indicate a statistical difference from the uninjected control.

**RNA-sequencing and analysis**

Libraries were generated using the Illumina Tru-Seq Low Sample protocol (Illumina), quantified using the KAPA Illumina library quantification kit (Kapa Biosystems), and checked for quality using a 2100 Bioanalyzer (Agilent Technologies, Inc.). The libraries have been submitted for 100bp paired-end sequencing on a HiSeq 4000 at UC Berkeley’s Functional Genomics Laboratory. Resulting data sets will be analyzed using a standard pipeline, as published (Kjolby and Harland, 2016). Reads will be mapped to the *X. laevis* genome, version 9.1, using TOPHAT (Trapnell et al., 2009). HTSeq (Anders et al., 2015) will be used to count reads, and DESeq (Anders and Huber, 2010) will be used to analyze differential gene expression.
Figure 3.1 *sall1* and *sall4* are expressed in the neural plate and neural tube

RNA *in situ* hybridization was performed on unmanipulated embryos using probes complementary to *Xenopus laevis sall1* or *sall4* at several stages through neurulation. These two genes are broadly expressed early in development, and take on distinct and more restricted expression patterns as neural development proceeds. Representative embryos are shown, with quantification in the lower right-hand corner of each panel. Dorsal view; anterior up, posterior down.
Figure 3.2 *sall1* and *sall4* are required for proper neural marker gene expression

Embryos were injected into the animal, dorsal, right cell at the 4-cell stage with 20ng morpholino (MO), then subjected to RNA *in situ* hybridization using probes complementary to markers of the neural plate. SB = splice-blocking, morpholino complementary to sequence including the exon1-intron1 boundary of *sall1* or *sall4*. TB = translation-blocking, morpholino complementary to sequence including the start codon of *sall1* or *sall4*. Representative embryos are shown, with quantification for this single experiment in the lower right-hand corner of each panel. Further quantification can be found in Figure 3.7b. Dorsal view; anterior up, posterior down.
Figure 3.3  *sall1* and *sall4* are required for neural differentiation

Embryos were injected into the animal, dorsal, right cell at the 4-cell stage with 20ng morpholino (MO), then subjected to RNA *in situ* hybridization using a probe complementary to neural beta-tubulin (*tubb2b*). Representative embryos at the indicated stages are shown, with quantification in the lower right-hand corner of each panel. Dorsal view; anterior up, posterior down.
Figure 3.4 *sall1* and *sall4* are required for neural tube closure

Embryos were injected at the 4-cell stage with 20ng morpholino (MO) per injected blastomere, then imaged at stage 16 (top three rows) or stage 24 (bottom row). Representative embryos are shown, with quantification for these individual experiments in the lower right-hand corner of each panel. Further quantification can be found in Figure 3.9. (A-D') Injected into the animal, dorsal, right cell. Top row, splice-blocking morpholinos; bottom row, translation-blocking morpholinos. Top and bottom rows are siblings from the same day of injection. (F-I) Injected into animal, dorsal, right and left cells. Splice-blocking morpholinos were used in these experiments. (I-M) Injected splice-blocking morpholino(s) into the animal, dorsal, right cell, and raised to stage 24. All *sall* morphants exhibit severe open neural tube phenotypes; the double morphant additionally exhibits a mild blastopore closure defect. Dorsal view; anterior up, posterior down.
Figure 3.5 *sall1* and *sall4* are required for hingepoint cell formation

Embryos were injected into the animal, dorsal, right cell at the 4-cell stage with 20ng morpholino (MO), sectioned transversely on a vibratome, and stained for actin using phalloidin-AlexaFluor488 (green), microtubules using anti-tubulin AlexaFluor-555-conjugated antibody (red), and nuclei using DAPI (blue). Top row: control morpholino and splice-blocking morpholinos; bottom row: translation-blocking morpholinos. Each pair of panels includes a larger panel showing the neural plate, notochord, and some of each somite (e.g., panel A); and a smaller panel of the same section showing a close-up of the neural plate (e.g., panel A’). Representative embryos are shown, with quantification for this single experiment in the lower right-hand corner of each large panel. Scale bar = 50μm in all panels. Further quantification can be found in Figure 3.10. Transverse cross-sectional view; dorsal up.
Figure 3.6 *pou5f3* genes are derepressed in *sall1* and/or *sall4* morphants

Embryos were injected into the animal, dorsal, right cell at the 4-cell stage with 20ng morpholino (MO), then subjected to RNA *in situ* hybridization using probes complementary to members if the *pou5f3* gene family. Splice-blocking morpholinos were used in this experiment. Representative embryos are shown, with quantification in the lower right-hand corner of each panel. Dorsal view; anterior up, posterior down. Quantitative real-time PCR data is shown for the same experiment, performed on a different batch of non-sibling embryos on a different day. Asterisks show statistical significance based on Tukey's honestly significantly different test after analysis of variance; “n.s.” indicates a non-significant difference compared to uninjected controls. qPCR primers are listed in Appendix II.
Figure 3.7a *pou5f3* gene overexpression causes neural anteroposterior patterning defects similar to those in *sall1* and/or *sall4* morphants

Embryos were injected into the animal, dorsal, right cell at the 4-cell stage with *pou5f3* family member mRNA (except uninjected controls), then subjected to RNA *in situ* hybridization using probes complementary to markers of the neural plate. Representative embryos are shown, with quantification for this single experiment in the lower right-hand corner of each panel. Compare to Figure 3.2; further quantification can be found in Figure 3.7b. Dorsal view; anterior up, posterior down.
Figure 3.7b Neural plate marker phenotypes in *sall* morphants and *pou5f3*-overexpressing embryos

Quantification of all experiments to assess neural plate marker gene expression in unilateral *sall1* and/or *sall4* morphant or *pou25*, *pou60*, or *pou91*-overexpressing embryos. See Figure 3.2 and Figure 3.7a for representative images. Boxed numbers show percentage with the most common phenotype.
Figure 3.8  *pou5f3* gene overexpression causes neural differentiation defects similar to those in *sall1* and/or *sall4* morphants

Embryos were injected into the animal, dorsal, right cell at the 4-cell stage with *pou5f3* family member mRNA (except uninjected controls), then subjected to RNA *in situ* hybridization using a probe complementary to neural beta-tubulin (*tubb2b*). Representative embryos are shown, with quantification in the lower right-hand corner of each panel. Compare to Figure 3.3. Dorsal view; anterior up, posterior down.
Figure 3.9 *pou5f3* gene overexpression causes neural tube closure defects similar to those in *sall1* and/or *sall4* morphants

Embryos were injected at the 4-cell stage with *pou5f3* family member mRNA (except uninjected controls), then imaged at stage 16. Representative embryos are shown, with quantification for these individual experiments in the lower right-hand corner of each panel. Dorsal view; anterior up, posterior down. Graph: quantification of all experiments to assess neural tube closure phenotype in unilateral *sall1* and/or *sall4* morphant or *pou25-, pou60-,* or *pou91*-overexpressing embryos. Boxed numbers show percentage with the most common phenotype.
Figure 3.10 *pou5f3* gene overexpression causes hingepoint cell formation defects similar to those in *sall1* and/or *sall4* morphants

Embryos were injected into the animal, dorsal, right cell at the 4-cell stage with *pou5f3* family member mRNA (except uninjected controls), sectioned transversely on a vibratome, and stained for actin using phalloidin-AlexaFluor488 (green), microtubules using anti-tubulin AlexaFluor-555-conjugated antibody (red), and nuclei using DAPI (blue). Each pair of panels includes a larger panel showing the entire neural plate, notochord, and some of each somite; and a smaller panel of the same section showing a close-up of the neural plate. Representative embryos are
shown, with quantification for this single experiment in the lower right-hand corner of each large panel. Scale bar = 50μm in all panels. Transverse cross-sectional view, dorsal up. Graph: quantification of all experiments to assess neural tube closure and actin accumulation phenotypes in unilateral \textit{sall1} and/or \textit{sall4} morphant or \textit{pou25-}, \textit{pou60-}, or \textit{pou91}-overexpressing embryos. See Figure 3.5 for representative \textit{sall} morphant images. Boxed numbers show percentage with the most common phenotype.
References


Kurth, T. (2005). A cell cycle arrest is necessary for bottle cell formation in the early Xenopus gastrula: Integrating cell shape change, local mitotic control and


CRISPR-Cas9 targeting in vivo. Nat Meth 12, 982–988.


Smith, J.C., and Howard, J.E. (1992). Mesoderm-inducing factors and the control of


Appendices

Appendix I: Morpholino oligonucleotide sequences

<table>
<thead>
<tr>
<th>Target transcript</th>
<th>Morpholino sequence</th>
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<tr>
<td><em>strabismus</em> (stbm)</td>
<td>5' CGTTGGCGGATT'TGGGTCCCCCGA 3'</td>
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<tr>
<td><em>celsr</em></td>
<td>5' GGGAGCCCTACACATCGGGCA 3'</td>
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<tr>
<td><em>inversin</em> (inv)</td>
<td>5' GAAACCTGAGGCTGCTACTCATA 3'</td>
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<tr>
<td>sal1 (splice-blocking morpholino)</td>
<td>5' GTGCCACTTACCAGACTCAGGC 3'</td>
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<td>sal4 (splice-blocking morpholino)</td>
<td>5' AGAGAAAGGACGTGGGACCTACCA 3'</td>
</tr>
<tr>
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<tr>
<td>sal4 (translation-blocking morpholino)</td>
<td>5' GCCAATTATCCCTTTCTCCACC 3'</td>
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Appendix II: qRT-PCR primer sequences

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<th>Gene</th>
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<th>Reverse primer sequence</th>
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<td>eef1a1</td>
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<td>5'-GGACACCAGTTCCACACGA-3'</td>
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<td>5'-GGACCTAAAGGCGCAGATAG-3'</td>
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<tr>
<td>pou91</td>
<td>5'-ACTTATTTGCCGGTCCTCCT-3'</td>
<td>5'-CCGATTACAGATCAGTGCT-3'</td>
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