INVESTIGATION OF AXIN2 IN ZEBRAFISH (DANIO RERIO) DEVELOPMENT AND ITS ROLE IN CANONICAL WNT SIGNALING

by

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Guelph, Ontario, Canada

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ABSTRACT

INVESTIGATION OF AXIN2 IN ZEBRAFISH (DANIO RERIO) DEVELOPMENT AND ITS ROLE IN CANONICAL WNT SIGNALING

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Canonical Wnt signaling is involved in many aspects of development including axis specification and anterior-posterior neuroectoderm formation during vertebrate embryogenesis. Axin2, a homologue of Axin1, is thought to have a similar regulatory role within the cell, but differences in their expression and binding partners suggest Axin2 is not completely redundant with Axin1. To better understand Axin2 in canonical Wnt signaling, I utilized several approaches to explore its expression and function. In the zebrafish embryo, I found Axin2 is expressed in known active domains of Wnt signaling, suggesting an inducible regulatory role. Additionally, canonical Wnt signaling was sufficient and necessary to induce Axin2 expression and Axin2 was sufficient and necessary to inhibit Wnt signaling. As Wnt signaling is important in development and its dysregulation has been implicated in diseases such as colorectal cancer, this study helps advance our understanding of how Wnt signaling regulates itself through the use of negative feedback inhibitors, such as Axin2.
ACKNOWLEDGEMENTS

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DECLARATION OF WORK PERFORMED

All of the work in this thesis was performed by Whitney Lum, with the exception of cloning the axin2-myc construct used for axin2 overexpression (Figure 15, Figure 16) and axin2MO verification (Figure 17), which was performed by Nicholas Fortino.
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<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>Boz</td>
<td>Bozozok</td>
</tr>
<tr>
<td>Cer</td>
<td>Cerberus</td>
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<tr>
<td>Chd</td>
<td>Chordin</td>
</tr>
<tr>
<td>CK1</td>
<td>Casein kinase1</td>
</tr>
<tr>
<td>CRD</td>
<td>Cysteine-rich domain</td>
</tr>
<tr>
<td>dpf</td>
<td>days post fertilization</td>
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<tr>
<td>DIG</td>
<td>Digoxigenin</td>
</tr>
<tr>
<td>DIX</td>
<td>Dishevelled-axin</td>
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<tr>
<td>Dkk</td>
<td>Dickkopf</td>
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<tr>
<td>Dsh/Dvl</td>
<td>Dishevelled</td>
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<tr>
<td>En</td>
<td>Engrailed</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>Frz</td>
<td>Frizzled</td>
</tr>
<tr>
<td>FrzB</td>
<td>Frisbee</td>
</tr>
<tr>
<td>Gsc</td>
<td>Goosecoid</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase 3β</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylases</td>
</tr>
<tr>
<td>Hh</td>
<td>Hedgehog</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse Radish Peroxidase</td>
</tr>
<tr>
<td>hpf</td>
<td>hours post fertilization</td>
</tr>
<tr>
<td>Int1</td>
<td>Intigration1</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LEF</td>
<td>Lymphocyte enhancer-binding factor</td>
</tr>
<tr>
<td>LRP</td>
<td>Lipoprotein receptor-related protein</td>
</tr>
<tr>
<td>Mbh</td>
<td>Mid-brain hindbrain</td>
</tr>
<tr>
<td>Mbl</td>
<td>Masterblind</td>
</tr>
<tr>
<td>Mkp3</td>
<td>MAP kinase phosphatase3</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>---------------------------------------------------</td>
</tr>
<tr>
<td>MO</td>
<td>Morpholino oligonucleotides</td>
</tr>
<tr>
<td>Mom</td>
<td>More mesoderm</td>
</tr>
<tr>
<td>Nkd</td>
<td>Naked cuticle</td>
</tr>
<tr>
<td>Nkd1</td>
<td>Naked cuticle homologue1</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate Buffered Saline with 0.1% Tween 20</td>
</tr>
<tr>
<td>PCP</td>
<td>Planar cell polarity</td>
</tr>
<tr>
<td>Por</td>
<td>Porcupine</td>
</tr>
<tr>
<td>RGS</td>
<td>Regulator of G protein signaling</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse Transcriptase</td>
</tr>
<tr>
<td>SSC</td>
<td>Sodium Chloride-Sodium Citrate Buffer</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>sFRP1</td>
<td>Secreted frizzle related protein1</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic hedgehog</td>
</tr>
<tr>
<td>Slb</td>
<td>Silberblick</td>
</tr>
<tr>
<td>TCF</td>
<td>T cell-specific transcription factor</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated Region</td>
</tr>
<tr>
<td>Wg</td>
<td>Wingless</td>
</tr>
<tr>
<td>Wf</td>
<td>Wingful</td>
</tr>
<tr>
<td>WMISH</td>
<td>Whole mount in situ hybridization</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless + Intigration1</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
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CHAPTER 1: INTRODUCTION
Cellular signaling pathways are a fundamental part of animal development, allowing for specific gene expression within specific tissues to create and maintain the developing body plan. Regulation of these signaling pathways throughout an organism’s life is therefore crucial to coordinate cellular activities during development. With normal development relying heavily on signaling pathways functioning correctly in specific tissues at particular times in development, multiple levels of regulation have evolved to ensure proper spatial and temporal gene expression. Moreover, pathway specificity can be achieved with the added complexity of multiple ligand and receptor families, as well as various transcriptional activators and repressors. While regulation and specificity are crucial to normal development, this complexity can also result in disaster when signaling goes awry. Instances of dysregulation within signaling pathways have been observed in many different species throughout development and have been further implicated in developmental defects and diseases, including cancer (Lustig et al., 2002; Frodde and Brabletz, 2007). The Wnt signaling cascade is no exception, as it has been observed to play a central role in normal development and instances of dysregulation can lead to disease including cancer.

Various signaling pathways must work together to direct normal patterning and proper development. Fibroblast growth factor (FGF), transforming growth factor β (TGFβ), bone morphogenetic protein (BMP), sonic hedgehog (Shh) and Wnt signaling are just a few signaling pathways which work together during early development to pattern the embryo. FGF signaling is involved in early patterning of mesoderm, which later impacts gastrulation movements and neural induction, ultimately leading to
dorsal/ventral patterning of the embryo (LaBonne and Whitman 1994; Cornell and Kimelman 1994). TGFβ signaling also plays an important role in ventral differentiation of tissues, regulating cellular processes including cell growth, cell differentiation and apoptosis (Khalsa et al., 1998). Additionally, TGFβ comprises a superfamily of ligands that includes Activin, Nodal, and BMP (Khalsa et al., 1998). BMP consists of a sub-group of growth factors involved in neural induction, neural crest formation and subsequent antagonism of TGFβ signaling (Lim et al, 2000). While FGF, TGFβ and BMP, amongst others, work in partnership to pattern the dorsal-ventral axis and induce neural expression, Shh plays a role in patterning of the neural tube and limbs, as well as anterior/posterior patterning (Echelard et al., 1993).

Wnt signaling activity is also active throughout both vertebrate and invertebrate development, and influences processes including cell polarity, cell specification and cell fate (reviewed in Croce and McClay, 2008). Maternal Wnt signaling is one of the first pathways activated during vertebrate embryogenesis, following fertilization and cortical rotation of the egg cytoplasm (Miller et al., 1999). Specifically within zebrafish (Danio rerio) embryos, known genetic targets of maternal Wnt signaling are expressed within the dorsal organizer as early as 3 hours post fertilization (hpf) (Fekany et al., 1999; Shimizu et al., 2000; Solnica-Krezel et al., 2001). As zygotic gene expression is activated, Wnt ligands can act as morphogens allowing for cell-to-cell communication through interactions between ligand and cell surface receptors. In zebrafish, this is observed in the specification of anterior and posterior neuroectoderm at 7 hpf via activation or inhibition of Wnt signaling (Van Raay et al., 2007; Klaus and Birchmeir, 2008). To date,
many advances have been made regarding our understanding of when discrete Wnt signaling events occur throughout development and what proteins are active during the signaling cascade to inducing target gene expression; however, much remains unknown about its regulation and maintenance. In addition to early embryogenesis and axis specification, it has been suggested that colorectal cancers arise in stem cells with overactive Wnt signaling within intestinal crypts (Reya and Clevers, 2005; van Es et al., 2005). Therefore, acquiring a more comprehensive understanding of Wnt signaling and its regulation at multiple levels could prove helpful in attaining a better understanding of how to re-establish homeostatic levels of expression in dysregulated cells.

The history of Wnt signaling

Wnt proteins are uniquely defined by their protein sequence rather than by their common functional properties. The Wnt family is comprised of 19 family members in mammals and includes a group of secreted glycoproteins which contain a highly conserved pattern of 23-24 cysteine residues (Willert et al., 2003; Logan and Nusse, 2004). Though Wnt signaling has been shown to be involved in many cellular processes, all Wnt proteins have been characterized to activate one of three main pathways: the Wnt/β-catenin pathway, the Wnt/Planar Cell Polarity (PCP) pathway, or the Wnt/Ca$^{2+}$ pathway (Widelitz, 2005). Collectively, the latter two pathways are known as the non-canonical Wnt signaling pathway, while the Wnt/β-catenin pathway is known as the canonical Wnt signaling pathway. This study focuses on the canonical Wnt signaling cascade and hereafter this pathway will be referred to as Wnt signaling.
The first Wnt gene was discovered in mice (known as integration1 (int1)) in 1982, with its homologue wingless (wg) discovered in Drosophila soon afterwards (Nusse and Varmus, 1982; Cabrera et al., 1987). Though not initially discovered in Drosophila, mechanisms of Wnt signaling were originally understood in that organism through the observation of several Drosophila mutant phenotypes that were consistent with mutations in wg (Nusslein-Volhard and Wieschaus, 1980; Wieschaus and Riggleman, 1987; Perrimon et al., 1989). It was observed that mutations in wg commonly result in defects in segmentation and wg was later understood to be required for the maintenance of hedgehog (Hh) and engrailed (En) expression in adjacent parasegment boundaries within the cuticle (Wieschaus and Riggleman, 1987; Perrimon et al., 1989). Additionally, mutations in porcupine (por), disheveled (dsh), and armadillo (arm - Drosophila homologue of β-catenin) all seemed to lead to segment polarity defects as well (Nusslein-Volhard and Wieschaus, 1980). It was later found that these segment polarity developmental phenotypes in Drosophila were all associated with mutations within components of what is now termed the Wnt signaling pathway, a name which encompasses both wg and int (Klaus and Birchmeir, 2008).

Recently, the nematode Caenorhabditis elegans has also emerged as a model organism, in addition to Drosophila, for the study of Wnt signaling. There are five known Wnt genes within C. elegans, with the Wnt ligand ‘more mesoderm2’ (mom-2) implicated in fate choice and the establishment of polarity within the embryo (Rocheleau et al., 1997; Prasad and Clark, 2006). Furthermore, mom-1 has been found to encode the homologue to Drosophila por, while mom-5 belongs to the Frizzled family of cell-surface
proteins involved in Wnt signaling as a cell surface receptor (Bhanot et al., 1996; Rocheleau et al., 1997). In mice, a considerable number of known wnt loss-of-function mutations have been generated resulting in axial patterning mutants much like those found in Drosophila and C. elegans. However, additional phenotypes which are not limited to segment polarity have also emerged with these knockdown studies. For example, the targeted inactivation of wnt7A results in mice with ventralized limbs, including dorsal tendons which mirror ventral tendon structures, indicating that wnt7A is required as a dorsalizing signal for normal dorsal-ventral polarity in limbs (Parr and McMahon, 1995). Taken together, when homologues of the pathway are mutated, similar patterning defects in Drosophila, C. elegans and mouse models show conservation in the Wnt signaling cascade. As such, work in these various animal models has begun to slowly elucidate the conserved molecular mechanism of Wnt signaling and the key signaling proteins involved in this cascade.

Canonical and Non-canonical Wnt Signaling

Non-canonical Wnt signaling is less understood than canonical and acts in a manner independent of β-catenin to influence developmental processes such as cell adhesion, cell polarity, and cell convergence and extension movements (reviewed in Widelitz, 2005). In particular, within non-canonical Wnt signaling, the PCP pathway has been characterized to not only direct cell movement, but also play a role in actin stabilization and microtubule arrangement during processes such as angiogenesis, neurulation and gastrulation (Heisenberg et al., 2000; De Calisto et al., 2005; Wallingford 2005; Ju et al., 2010). On a molecular level, Wnt ligands, the frizzled
receptor (Frz) and the cellular scaffolding protein Dsh are common to both canonical and non-canonical signaling, whereas proteins such as the small GTPases RhoA and Rac, as well as c-Jun N-terminal kinase (JNK) are unique to the non-canonical pathway (De Calisto et al., 2005). Studies involving non-canonical Wnt signaling were initiated in *Drosophila*, but more recently have expanded to *Xenopus* (Wallingford, 2005) and zebrafish primarily through the observation of mutant phenotypes (Sepich et al., 2000; Topczewski et al., 2001; Jessen et al., 2002). For example, one of the earliest loss-of-function mutations identified in zebrafish was silberblick (*slb*), which results in cyclopia and other midline defects (Heisenberg et al., 1997). It was later found that *slb* encodes the Wnt11 ligand that is required for cells to undergo correct extension movements, which underlie vertebrate gastrulation (Heisenberg et al., 2000). In addition, *trilobite* and *knypek* mutants both result in perturbed convergence and extension movements, resulting in embryos that are very short in length (Sepich et al., 2000; Topczewski et al., 2001; Jessen et al., 2002).

In contrast, canonical Wnt signaling occurs through β-catenin and is involved in a broad range of processes such as cell proliferation, embryonic axis specification and anterior-posterior neuroectoderm formation (Moon et al., 1997; Cadigan and Nusse, 1997; Xe et al., 1997; Tao et al., 2005; Van Raay et al., 2007). Within vertebrate development, the loss of a single protein within the canonical Wnt signaling cascade or target gene can result in phenotypes that range from kidney and central nervous system (CNS) defects to embryonic lethality (Herzlinger et al., 1994; Ikeya et al., 1997; Liang et al., 2003). In zebrafish and frog, the first canonical Wnt signaling event is initiated under
maternal control at the point of fertilization and continues to be active at discrete time points throughout embryonic development (Miller et al., 1999; Tao et al., 2005). Additionally, canonical Wnt signaling has also been observed within adult cells to maintain tissue homeostasis (Nusse 2005; Katoh and Katoh, 2007). Given the large number of Wnt ligands with such a wide variety of functions, it is of no surprise that canonical Wnt signaling influences a large number of developmental decisions over the lifetime of an organism from embryo to adult, with instances of its dysregulation leading to diseases such as cancer. Although the fundamentals of Wnt signaling are steadily being delineated, a clear understanding of the regulation of the pathway has yet to be achieved.

Canonical Wnt Signaling: Resting State

In the absence of a Wnt ligand (i.e. the pathway is inactive), a constitutively active destruction complex composed of two scaffolding proteins, Axin1 and adenomatous polyposis coli (APC), and a kinase, glycogen synthase kinase 3β (GSK3β), binds to and phosphorylates β-catenin at its N-terminus. Specifically, Axin1 and APC enable GSK3β to effectively co-localize and phosphorylate β-catenin (Barker and Clevers 2000; Jho et al., 2002; Reya and Clevers 2005; Widelitz, 2005). Phosphorylation of β-catenin subsequently targets cytoplasmic β-catenin for ubiquitin mediated degradation (Figure 1) (Kishida et al., 1998; Huelsken and Behrens, 2002; Reya and Clevers 2005). It is this ubiquitin-mediated degradation of β-catenin by the destruction complex which keeps cytoplasmic β-catenin at low levels, maintaining the signaling cascade in its resting state. As a result, constant degradation of β-catenin by Axin1, APC and GSK3 is crucial
in the inhibition of Wnt-mediated signaling. The function of Axin1 has recently been revealed through both in vitro and in vivo studies as an important regulator of Wnt signaling (reviewed in Polakis, 2000). In mice, mutations in axin1 result in embryonic lethality at embryonic day 9.5 (Chia and Costantini, 2005), while in zebrafish the axin1 mutant masterblind (mbl), displays less severe phenotypes and shows defects in anterior-posterior patterning of the brain (van de Water et al., 2001). The mbl mutant carries a mutation in the GSK3β binding domain of axin1, resulting in the accumulation of β-catenin. This leads to constitutive activation of the pathway and alterations in cell fate determination within the prospective forebrain (van de Water et al., 2007). With Axin1 expressed ubiquitously throughout the developing embryo along with its constitutive role in degrading β-catenin, it is clear that Axin1 plays a vital role in Wnt signaling regulation.

While constant ubiquitination and degradation of β-catenin is occurring in the cytoplasm, within the nucleus, T cell-specific transcription factor/lymphocyte enhancer-binding factor (TCF/LEF) transcriptional repressors inhibit transcription of Wnt genes by forming a complex with Groucho (Arce et al., 2009) (Figure1). Groucho mediates interactions between histone deacetylases, which repress transcriptional activation (Arce et al., 2009).
Figure 1: Canonical Wnt signaling at rest. In the absence of a Wnt ligand, GSK3β within the destruction complex targets cytosolic β-catenin for phosphorylation and subsequent ubiquitin mediated degradation. Within the nucleus, TCF/LEF and Groucho act together as a repressor, preventing transcription of Wnt target genes in the absence of Wnt stimulation.
**Canonical Wnt Signaling: Active State**

Activation of Wnt signaling occurs by the binding of a Wnt ligand to its appropriate Frz receptor at the plasma membrane. Frz receptors contain seven-transmembrane domains and an extracellular N-terminal cysteine-rich domain (CRD) (Bhanot *et al.*, 1999). It is the binding of Wnt ligands to the Frz CRD domain and subsequent recruitment of lipoprotein receptor-related protein (LRP) that initiates signaling (Itasaki *et al.*, 2003). Upon trimeric complex formation between Wnt ligand, Frz and LRP, Dsh is recruited to the membrane where it interacts with Frz via its C-terminal domain and becomes activated (Giles *et al.*, 2003; Klaus and Birchmeier, 2008). Dsh plays a unique role in Wnt signaling, acting at the crossroads between canonical and non-canonical Wnt signaling cascades (Van Raay *et al.*, 2007); however, its exact function in differentiating between pathways remains elusive. In spite of this, it is known that during active Wnt signaling, Dsh disrupts the destruction complex by binding Axin1, in turn preventing β-catenin from being phosphorylated by GSK3β. Unphosphorylated β-catenin accumulates within the cytosol (Li *et al.*, 1999), translocates into the nucleus where it associates with TCF/LEF to relieve transcriptional repression and activate transcription of Wnt-specific target genes (Figure 2) (Boutros and Mlodzik, 1999; Huelsken and Behrens, 2002). Repression is relieved through the displacement of Groucho from TCF/LEF and thus recruitment of histone acetylases (Hecht *et al.*, 2000). Mutations that disrupt destruction complex formation result in stabilized β-catenin accumulation and are known to cause cancer (Yan *et al.*, 2001; Reya and Clevers, 2005; O’Brien *et al.*, 2007). This underscores the importance in the regulation of β-catenin stability, placing it at the epicenter of the canonical Wnt signaling cascade.
Figure 2: Active canonical Wnt signaling. A Wnt ligand binds cell surface receptor Frz, subsequently recruiting co-receptor LRP and thus activating the Wnt signaling cascade. This binding causes the recruitment of intracellular Dsh to interact with the Frz receptor and Axin1 within the destruction complex. Dsh interaction with Axin1 disrupts the destruction complex, eliminating phosphorylation of β-catenin by GSK3β and subsequent ubiquitin-mediated degradation. Accumulation of cytosolic β-catenin allows for its translocation into the nucleus where it binds TCF/LEF, activating transcription of Wnt target genes.
Extracellular and Intracellular Regulation of Canonical Wnt Signaling

Regulation can occur at multiple levels within a signaling cascade, both in the extracellular and intracellular environment. Regulation is required for temporal and spatial patterning as well as to control the duration and intensity of a signal. The Wnt signaling cascade is no exception, with both extracellular and intracellular components that regulate its activity throughout development. Extracellular regulators can be induced, for example, by neighboring cells in a specific or temporal manner, while intracellular regulators, typically negative feedback regulators, provide a mechanism for intensity and durational control of a signal.

Extracellularly, dickkopf (Dkk) is a major player in temporal and spatial regulation of Wnt signaling (Shinya et al., 2000; Niida et al., 2004). Dkk1 inhibits dorsal Wnt signaling by binding LRP5/6 with high affinity. LRP5/6 is a transmembrane co-receptor that is believed to form a trimeric complex with the Wnt and Frz receptor to initiate Wnt signaling (Itasaki et al., 2003). The interaction between Dkk1 and LRP6, however, initiates the internalization of LRP6, thus preventing Wnt signaling from being initiated (Shinya et al. 2000; Bafico et al. 2001). Other secreted proteins such as cerberus (Cer) and frisbee (FrzB) have also been found to directly bind specific Wnt ligands rather than transmembrane receptors to inhibit the initiation of Wnt signaling (Hoang et al. 1996; Piccolo et al., 1997; Wang et al., 1997; Bafico et al. 2001). For example, secreted frizzled related protein1 (sFRP1) selectively binds specific Wnt ligands by its cysteine-rich domain, which resembles the ligand binding domain of the Frz receptor (Kim et al. 2007). Thus, while the action of Dkk1 is an example of general spatial and temporal
While temporal regulation and specificity of Wnt signaling can be accomplished extracellularly, intracellular regulation occurs as well. Intracellular negative feedback regulation is a common tool used to restrict the duration and intensity of a signal and typically acts in an inducible manner to allow for tight control of signaling (Miller et al., 1999). There are many putative inducible intracellular negative feedback regulators within the Wnt signaling pathway, including wingful (Wf), naked cuticle (Nkd) in flies, Nkd1 in vertebrates, and more recently, Axin2 (Van Raay et al., 2007; Niida et al., 2004; Gerlitz and Basler, 2002; Yan et al. 2001; Jho et al. 2002; Nakamura et al., 1998). Wf was first identified to play a role in imaginal disc and bristle formation as well as segmentation in Drosophila (Gerlitz and Basler, 2002). Mutations in wf result in a wg gain-of-function phenotype and further studies lead to the identification of wf as a Wg inducible negative regulator acting at the level of Frz receptor or LRP co-receptor (Gerlitz and Basler, 2002). Interestingly, there is currently no known vertebrate homologue of wf. Nkd/Nkd1 has been well established as a negative feedback regulator of Wnt signaling and studies in both flies and vertebrates have demonstrated that nkd/nkd1 is not only a target of Wnt signaling, but necessary and sufficient to antagonize the Wnt signaling cascade at multiple stages throughout development (Rousset et al., 2001; Yan et al., 2001; Van Raay et al., 2007). This has lead to the hypothesis that Nkd1 is an obligate and universal target of the Wnt signaling pathway (reviewed in MacDonald et al., 2009). Nkd/Nkd1 antagonism unlike wf, however, acts at the level of Dsh within
the cytoplasm to inhibit the accumulation of nuclear β-catenin, with Dsh and β-catenin acting in a competitive manner for binding to Nkd1 (Van Raay et al., 2011). Thus, nkd1 and wf are good examples of intracellular inducible negative feedback regulators of Wnt signaling that control the intensity and/or duration of a signal.

**Axin2 – an inducible negative regulator of Wnt signaling**

As alluded to previously, Axin1 plays a crucial role in constitutive intracellular regulation of Wnt signaling by acting as a scaffolding protein within the destruction complex that allows GSK3β to co-localize with β-catenin. As a result, β-catenin is phosphorylated, targeting it for ubiquitin-mediated degradation and leaving Wnt signaling in a resting state (Heisenberg et al., 2001). The Axin1 paralogue Axin2, known as Conductin in mice, shares 44% sequence homology with Axin1 (Figure 3). There are several conserved domains including a regulator of G protein signaling (RGS) domain (critical in binding with APC) and a disheveled-axin intracellular domain (DIX) (Chia and Costantini 2005) (Figure 3). While there have been fewer studies with Axin2 compared to Axin1, preliminary findings indicate that like Axin1, Axin2 contains binding domains for β-catenin and GSK3β, suggesting that Axin1 and Axin2 share similar inhibitory roles within the cell (Chia and Costantini, 2005). Indicative of these shared functions, both Axin1 and Axin2 can reduce cytosolic levels of β-catenin, which subsequently inhibits expression of Wnt target genes (Behrens et al., 1998; Furuhashi et al., 2001; Chia and Costantini, 2005). Interestingly, the expression of Wnts in areas such as the vertebrate primitive streak and dorsal neural tube during embryogenesis (Erter et al., 2001; Lekven et al., 2001) also overlaps with the expression of axin2, broadening the
role of *axin2* by suggesting it is a target of Wnt signaling. Furthermore, *in vitro* induction of Axin2 mRNA expression in the presence of Wnts, as well as TCF/LEF binding sites within the *axin2* promoter sequence, additionally supports the role of *axin2* as a target gene of Wnt signaling (Parr *et al.*, 1993; Jho *et al.*, 2002; Leung *et al.*, 2002).
**Figure 3**: Alignment of zebrafish *axin1* and *axin2* amino acid sequence. Red boxes identify identical residues. Numbering refers to the amino acid sequence of Axin1. Overlapping bars define conserved domains including RGS and DIX domains (Heisenberg et al., 2001).
However, while binding domains and an inhibitory role in Wnt signaling are shared between Axin1 and Axin2, there remain marked differences in their function and partnering interactions with other players of the Wnt signaling cascade throughout development. For example, interactions between Axin1 and Dsh/Dvl, as well as Axin1 and CK1/LRP seem to be involved in the mechanism of Axin1 inhibition on Wnt signaling; however, these interactions are not observed with Axin2 (Miller et al., 2009). Perhaps most interesting is that while mutations in axin1 result in embryonic lethality, mutations in axin2 display only cranial abnormalities (Yu et al., 2005). However, gene replacement studies have demonstrated that axin2, when put into the axin1 locus, can rescue axin1−/− embryonic lethality (Chia and Costantini, 2005). Conversely, in the presence of normal axin1 expression and the absence of axin2 expression through deletion, mice continue to exhibit skull abnormalities (Zeng et al., 1997; Yu et al., 2005). These findings suggest that neither axin1 nor axin2 can entirely substitute for one another and are therefore not completely functionally redundant despite their functionally similar inhibitory roles on Wnt signaling within the cell. A potential explanation for lack of complete redundancy in these two genes could be in their differing expression patterns. While axin1 is expressed ubiquitously, axin2 is expressed at specific developmental time points within certain tissues, displaying more restricted expression during early embryogenesis and organogenesis due to its inducible nature (Zeng et al., 1997; Jho et al., 2002; Yu et al., 2005). Additionally, in a recent study conducted by Qian et al. (2011), a novel gain-of-function mutant axin2comp displayed increases in Wnt activity at embryonic day 8.5 within the primitive streak suggesting a rare role for axin2 to act as an enhancer of Wnt signaling in a cell-type and time-dependent manner.
The differences between Axin1 and Axin2 highlight the differences between constitutive and induced negative regulation. Constitutive regulation is critical for normal signaling, while induced negative feedback regulation can work to buffer the cell against fluctuations in Wnt signaling and is not always required. Recently, interactions between Nkd1 and both Axin1 and Axin2 have been demonstrated suggesting a convergence between constitutive and induced negative feedback regulation (Miller et al., 2009). Additionally, Axin2 shares significant homology between human, mouse and zebrafish, implying a conserved role in multiple vertebrate systems (Figure 4). Together, these data demonstrate that regulation of Wnt signaling via negative feedback regulators is crucial, yet poorly understood and a better understanding of Axin2 and its role in Wnt signaling regulation is sought.
**Figure 4:** Alignment of human, mouse and zebrafish *axin2* amino acid sequence. Red boxes identify identical residues; blue boxes identify similarity with consensus sequence. Numbering refers to the amino acid sequence of Axin2.
Zebrafish (Danio rerio) as a model organism

With such well-characterized Wnt signaling events early in development, zebrafish has emerged as an excellent model organism to further elucidate the role of Axin2 as an inducible negative feedback regulator of Wnt signaling. Zebrafish first came to the forefront of research following its use in effective large-scale genetic screens, no longer limiting large-scale genetic screens to invertebrates and opening the doors to a vertebrate model (Driever et al., 1996). Early experiments involving zebrafish allowed for the identification of many mutations which affect physiology and early development (Driever et al., 1996; Shimoda et al., 1999; Dooley and Zon, 2000). Later, these mutations proved to be a valuable resource in identifying specific relationships between genes and function.

Despite the divergence of zebrafish and humans approximately 450 million years ago, conserved gene functions have been identified. Syntenic chromosomal regions between zebrafish and humans have also been observed, even though their last common ancestor was 450 million years ago (Kumar and Hedges, 1998). Furthermore, despite gene duplications, zebrafish and humans continue to have about the same number of chromosomes (zebrafish having 24 pairs and humans having 23 pairs) (Postlethwait et al., 2000). With such a high degree of homology between vertebrates, as well as remarkably conserved development, zebrafish can provide easy access and manipulation of one gene to provide insight into its homologue in other vertebrates, including humans.

Phenotypically, gene expression can easily be observed at multiple stages throughout zebrafish development through whole mount in situ hybridization (WMISH). Gene function is easily analyzed through the use of microinjected antisense morpholino
oligonucleotides (MO) (Kimmel et al., 1995; Postlethwait et al. 2000). Furthermore, zebrafish embryos are transparent, allowing for morphological observations to be made throughout development, beginning at the one-cell stage and continuing until gastrulation and organ development have occurred (Kimmel et al., 1995). Cellular development throughout this time can not only be manipulated and observed, but embryos can be harvested at critical time points throughout development for analysis. These observations can be made without having to dissect the organism as exemplified by the use of transgenic lines and reporter molecules (Dorsky et al., 2002). Also, with zebrafish reaching approximately 3-5cm in length and embryos only 1mm in diameter, hundreds of adults can be housed and maintained relatively easily and inexpensively (Kimmel et al., 1995). A single female can lay upwards of 300 eggs over several minutes and external fertilization allows for immediate collection and manipulation of embryos at the point of fertilization (Kimmel et al., 1995).

Wnt signaling in early zebrafish development

Early zebrafish development is generally categorized into eight stages: zygote, cleavage, blastula, gastrula, segmentation, pharyngula, hatching and early larva. The zygote stage begins at a newly fertilized egg and ends 72 hpf at the early larval stage where independent food seeking and avoidance behaviours begin to occur (Kimmel et al., 1995). The first cleavage occurs 45 minutes following fertilization and development continues with key stages such as gastrulation and segmentation occurring at 5.25 and 10 hpf, respectively (Kimmel et al. 1995). From the point of fertilization to 3.5 hpf the first Wnt signaling event occurs under maternal control. At this point, maternally induced
Wnt signaling leads to the expression of bozozok (boz)/dharma within the presumptive dorsal organizer (Fekany et al., 1999) (Figure 5A). Boz is a known target of maternal Wnt signaling and aids in the establishment of the dorsal organizer (Solnica-Krezel and Driever, 2001). For example, boz induces the expression of dorsal organizer specific genes such as gooscoid (gsc) and chordin (chd) in concert with Nodal and FGF signaling (Cornell and Kimelman 1994; Kawahara et al., 2000; Dixon Fox and Bruce, 2009).

Subsequently, between 3.5-5.5 hpf the second Wnt signaling event is initiated. That is, ventrolateral Wnt8 signaling commences and is required to restrict the ventro-lateral expansion of the organizer (Figure 5B). Along the putative ventro-lateral side of the embryo, it is maternal Nodal signaling which is inducing the expression of Wnt8 (Erter et al., 2001; Ramel and Lekven, 2004). Therefore, within the first 5.5 hours of fertilization there are two opposing Wnt signaling events occurring. There is the positive influence from maternal Wnt signaling occurring within the presumptive dorsal organizer to induce expression of dorsal organizer specific genes, and a negative influence from zygotic Wnt signaling to restrict ventro-lateral expansion of the dorsal organizer. Both signaling events are required to establish the primary dorsal-ventral axis. Between 5.5-7.5 hpf, the third Wnt signaling event occurs. At this stage, ventro-lateral Wnt8 is involved in the specification of anterior-posterior neuroectoderm formation (Erter et al., 2001; Lekven et al., 2001; Van Raay et al., 2007) (Figure 5C). Thus, within the first 7.5 hpf, there are at least 3 independent Wnt signaling events occurring and it is clear that their tight regulatory control is crucial for proper patterning to occur.
Figure 5: Wnt signaling from 3.5-7.5 hpf. (A) At 3.5 hpf, maternal Wnt signaling induces expression of goosecoid (gsc) in the presumptive dorsal organizer (purple). (B) At 5.5 hpf, zygotic Wnt signaling inhibits organizer from extending ventro-laterally. (C) At 5.5 hpf, zygotic induction of Wnt signaling establishes anterior and posterior neuroectoderm. Arrow indicates the prechordal plate. A, Vg, V and D indicate animal pole, vegetal pole, ventral and dorsal regions of the embryo, respectively (adapted from Van Raay et al. 2007). Embryos were staged according to Westerfield (Westerfield 2007).
Rationale and Specific Aims

The recent establishment of zebrafish as an effective model organism, along with well-characterized Wnt signaling events early in development, provides the potential to answer questions surrounding the regulatory mechanisms of Wnt signaling. In addition to the developmental impacts of this cascade, current findings suggest the involvement of canonical Wnt signaling in colon cancer development within stem cells of the intestinal crypt (Yan et al., 2001; Reya and Clevers, 2005; O’Brien et al., 2007). It has been shown that uncontrolled overactivation of canonical Wnt signaling results in tumor formation within the colon leading to cancer (Baker et al., 2009). As previously stated, investigation into the constitutive negative regulator Axin1 has been characterized in zebrafish and mouse models; however our understanding of Wnt induced negative feedback regulation on Wnt signaling in vivo is under-developed. Axin2 has emerged in the current literature as a potential candidate as a Wnt inducible negative feedback regulator, but currently there is no comprehensive expression profile demonstrating that axin2 is an obligate target of Wnt signaling or, as for many other Wnt target genes, axin2 expression is facultative. Furthermore, there is no evidence outside of the mouse model to demonstrate that Axin2 is a Wnt antagonist and differences between Axin1 and Axin2 function remain unresolved. As such, I have chosen zebrafish as a model system to characterize Axin2 in Wnt signaling. Specifically, I would like to establish if axin2 is an obligate target of active Wnt signaling in early development, and if the function of Axin2 is conserved. With this knowledge, I hope to provide a starting point for investigation into how Axin2 regulates Wnt signaling at a molecular level and build on clarification of how Axin1 and Axin2 function independently in regulating Wnt-mediated cell signaling.
Taken together, I seek to provide preliminary insight into how a signaling pathway that is critical for both development and disease regulates its own behavior. As such, I established three specific aims:

**Aim1:** To determine the spatial and temporal expression of *axin2* in zebrafish development.

**Aim2:** To determine if Wnt signaling is both necessary and sufficient to induce *axin2* expression.

**Aim3:** To determine if *axin2* is both necessary and sufficient to antagonize Wnt signaling.
CHAPTER 2: MATERIALS & METHODS
Fish Maintenance and Breeding

All adult zebrafish (*Danio rerio*) were housed at the Hagen Aquatics Facility at the University of Guelph. Zebrafish were maintained at 28.5°C (Westerfield 2007). Mating pairs were set up daily and consisted of 2 male and 2 female wildtype (AB* genetic backgrounds) adult zebrafish. Embryos were then collected directly following fertilization. Embryos were staged according to Westerfield (Westerfield 2007).

Aixin2 Cloning into pCR - BluntII - TOPO

a) RNA Extraction and cDNA synthesis

Total RNA was extracted from 10 wildtype embryos at 50% epiboly with TriPure TRIzol (Invitrogen, Burlington, ON, Canada), as described by Chomczynski and Sacchi (1987). For first strand cDNA synthesis 0.1 µg of dN₆ (random hexamers) (Invitrogen) and 1 mM of dNTP (Invitrogen) were added to 5 µg of resuspended RNA and incubated at 65°C for 10 minutes. Following incubation, 5X reverse transcriptase (RT) buffer, 0.1 µM MgCl₂, 0.2 µM DTT and 40 U RNase inhibitor (Invitrogen) was added to 10 µL of resuspended RNA containing dN₆ and dNTP in a 20 µL reaction and incubated at 25°C for 2 minutes. 200 U of Superscript II RT (Invitrogen) was then added to the sample prior to incubation at 25°C for 10 minutes followed by 42°C for 60 minutes for cDNA to be synthesized. Incubation at 70°C for 15 minutes terminated the reaction and 4 U of RNase H was added before incubating the sample at 37°C for 20 minutes to remove template mRNA. All reactions were carried out in a MyCycler Thermal Cycler (BioRad, Mississauga, ON, Canada).
b) PCR amplification and Cloning

Gene specific primers were designed to PCR amplify the axin2 open reading frame (ORF), the axin2 3’ untranslated region (UTR) and the axin2 5’UTR. All primers were designed in silico and synthesized by Invitrogen. Primers were resuspended in dH₂O into 100 µM stocks and further diluted with dH₂O to 10 µM working stocks to be used in PCR reactions (Table 1). Newly synthesized cDNA was added to a reaction mixture containing: 10 nM of forward and reverse primers, 1 U of GoTaq enzyme (Promega, San Luis Obispo, CA, USA) and 1 U Pfu enzyme (Fermentas, Burlington, ON, Canada) in a 50 µL reaction containing 1X Pfu buffer (Fermentas). Amplification conditions were as follows: 95°C for 5 minutes, followed by 30 cycles of 95°C for 50 s, 62°C for 30 s, 72°C for 2 min, and a final extension at 72°C for 10 minutes. Following PCR amplification, 10µL of all three constructs were resolved on a 1% agarose gel containing SYBR safe DNA gel stain (Invitrogen) to ensure each respective target sequence had been amplified.

PCR products were cloned directly into pCR-Blunt II-TOPO using a Zero Blunt TOPO PCR Cloning Kit (Invitrogen). Subsequently, selected colonies were grown overnight in 3 mL Luria Broth cultures containing 90ng kanamycin. Plasmids were purified with QIAprep Spin Miniprep Kit (Qiagen, Mississauga, ON, Canada) and sequenced (Advanced Analysis Center Genomics Facility, University of Guelph, ON, Canada). Plasmid DNA was used as a template for synthesis of either axin2 3’UTR, axin2 ORF or axin2 5’UTR antisense probes for in situ hybridization (Table 2).
addition to generating probe, *axin2* ORF was also cloned into pCS2+-Myc for overexpression analysis (see below).
Table 1. Primers used for cloning of Axin2 into TOPO Zero Blunt vector.

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Primer Sequence</th>
<th>bp</th>
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<tbody>
<tr>
<td><strong>Axin2 5’UTR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5’ – GAC TTT ACG GAC TGA ACT CA – 3’</td>
<td>20</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’ – TTC ACT GTC ATT GGC ACT CG – 3’</td>
<td>20</td>
</tr>
<tr>
<td><strong>Axin2 ORF</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5’ – GAC TTT ACG GAC TGA ACT CA – 3’</td>
<td>20</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’ – GAC ATC GAT GCT CCA GGA CA – 3’</td>
<td>20</td>
</tr>
<tr>
<td><strong>Axin2 3’UTR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5’ – GCC CTG CAG GGA ATT ACC CA – 3’</td>
<td>20</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’ – GAC ATC GAT GCT CCA GGA CA – 3’</td>
<td>20</td>
</tr>
</tbody>
</table>
**Probe Synthesis**

_Gsc, Chd_ and _Mkp3_ DNA constructs were provided by Dr. Solnica-Krezel lab (Vanderbilt University, Nashville, Tennessee, USA). 10 ng of DNA was digested overnight with a respective restriction endonucleases (New England Biolabs, Pickering, ON, Canada) in a 100 µL reaction at 37°C to linearize DNA (Table 2). Following digestion, 10 µL was resolved on a 1% agarose gel to ensure DNA had been fully digested and linearized. The remaining 90 µL was extracted with phenol chloroform/isoamylalcohol (Ambion, Streetsville, ON, Canada) and plasmid was ethanol precipitated at -20°C for 30 minutes. Precipitated DNA pellets were resuspended in 10 µL Rnase-free water. 1 µL of purified product was used to determine the concentration by spectrophotometer (BioRad) and 1 µL resolved on a 1% agarose gel containing SYBR safe DNA gel stain (Invitrogen) to confirm DNA recovery. The remaining product was stored at -20°C and used for probe synthesis.

Sense and antisense digoxigenin-labeled probes were synthesized from linear plasmid templates using MAXIscript – InVitro Transcription Kit (Ambion) and T7 or SP6 RNA polymerases (NEB) (Table 2). Transcription reactions were incubated for 2.5 hours in the following 20 µL reaction: 10x transcription buffer (Ambion), 1 µg of linear DNA template, 10x DIG RNA Labeling Mix (Roche), 40 U RNA polymerase (NEB) and nuclease free water (Ambion) to 20 µL. After incubation, 2 U TURBO DNase (Ambion) was added to remove DNA template. RNA was precipitated with 50 µM ammonium chloride and left to precipitate at -20°C for 30 minutes. RNA was resuspended in 20 µL of RNase free water. 1 µL of newly synthesized and labeled probes was resolved on a
1% agarose containing SYBR safe DNA gel stain (Invitrogen) to monitor synthesis, while concentrations of each sample were quantified using a spectrophotometer (BioRad). All probes were stored at -80°C until use.
Table 2: Restriction endonucleases and RNA polymerase used for various probe syntheses.

<table>
<thead>
<tr>
<th>Probe of Interest</th>
<th>Restriction Enzyme Used</th>
<th>Sense or Antisense</th>
<th>RNA Polymerase Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gsc</td>
<td>EcoRI</td>
<td>Antisense</td>
<td>T7</td>
</tr>
<tr>
<td>Chd</td>
<td>Xba</td>
<td>Antisense</td>
<td>T7</td>
</tr>
<tr>
<td>Axin2 3’UTR</td>
<td>NotI</td>
<td>Antisense</td>
<td>SP6</td>
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<tr>
<td>Axin2 3’UTR</td>
<td>BamHI</td>
<td>Sense</td>
<td>T7</td>
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<td>Axin2 5’UTR</td>
<td>NotI</td>
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<tr>
<td>Axin2 ORF</td>
<td>NotI</td>
<td>Antisense</td>
<td>T7</td>
</tr>
<tr>
<td>Nkd1</td>
<td>HindIII</td>
<td>Antisense</td>
<td>T7</td>
</tr>
</tbody>
</table>
Axin2 ORF Overexpression Construct

To epitope-tag Axin2, pCS2+ was used to fuse a Myc tag encoding sequence to the 5’ end of the Axin2 ORF. Axin2 ORF-TOPO and pCS2+-MT were digested with 30 U SstI (NEB) for 3 hours at 37°C in a 40 µL reaction containing 1X NEBuffer 4 (NEB). After digestion, samples were purified using a QIAquick PCR Purification Kit (Qiagen) and eluted in 30 µL of elution buffer. This 30 µL of purified DNA was re-digested with 60 U EcoRI (NEB) overnight at 37°C in 40 µL. 5 µL of digested DNA was resolved in a 0.8% agarose gel containing SYBR safe DNA gel stain (Invitrogen), to ensure complete digestion. The remaining 35 µL was excised from the gel and purified using a QIAquick Gel Extraction Kit (Qiagen). Following purification, vector and insert were ligated for 2 hours with T4 DNA ligase from Rapid DNA Ligation Kit (Roche, Laval, QC, Canada). 2 µL of the ligation was added to 50 µL of DH5α cells (Invitrogen) and cells were plated on LB agar containing 50 mg/mL ampicillin plates and incubated overnight at 37°C. Positive colonies were identified by sequencing (Advanced Analysis Center Genomics Facility, University of Guelph, ON, Canada) and all DNA constructs stored were at -20°C.

mRNA and Morpholino Oligonucleotide Preparation

For Specific Aims 2 and 3, capped mRNA for microinjection was synthesized using mMessage mMMachine kit (Ambion). Plasmids were digested for 3 hrs at 37°C and transcription reactions were incubated at 37°C for 4 hours (Table 3). Following transcription, RNA was purified using a MEGAclear purification of transcription reaction kit (Ambion) and diluted in RNase free water (Ambion) to a final concentration of
200ng/µL (axin2-myc), 10 ng/µL (ΔNβ-catenin), 400 ng/µL (nkd1) or 200 ng/µL (axin1) in 1% phenol red. 2 nL of diluted RNA was then microinjected into embryos at the one- to two-cell stage. Following injection, embryos were left to incubate at 28.5°C and later fixed in 4% paraformaldehyde (PFA) in 1X Phosphate Buffered Saline (PBS; 2.68 mM KCl, 1.47 mM KH$_2$PO$_4$, 136.89 mM NaCl, 8.10 mM Na$_2$HPO$_4$) at varying points of development for insitu hybridization.

The Axin2 morpholino oligonucleotide (MO) (GeneTools, Philomath, OR, USA) was designed as a 25 bp sequence complementary to the first 25 base pairs upstream of the first axin2 translational start site of the coding region. This 25 bp sequence (5’ – GAT CTG TTA GTG TCC TAT TCA TGG C – 3’) has little self-complementarity to avoid dimerization, and 40% GC content to ensure high target affinity. Axin2 MO was first re-suspended into 16ng/µL stocks using dH$_2$O and subsequently further diluted with dH$_2$O into 2ng/µL working stocks in 1% phenol red. Following dilution, 2-8 ng of Axin2 MO was injected into WT embryos at the one- to two-cell stage.
Table 3: Restriction enzyme and RNA polymerase used for various mRNA syntheses.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Restriction Enzyme Used</th>
<th>RNA Polymerase Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axin2</td>
<td>NotI</td>
<td>SP6</td>
</tr>
<tr>
<td>ΔNβ-catenin</td>
<td>NotI</td>
<td>SP6</td>
</tr>
<tr>
<td>Nkd1</td>
<td>NotI</td>
<td>SP6</td>
</tr>
<tr>
<td>Axin1</td>
<td>NotI</td>
<td>SP6</td>
</tr>
</tbody>
</table>
Western Blotting and Quantification

To validate MO knockdown of axin2 expression by western blot, embryos were microinjected with 200ng of Axin2-myc and co-injected with 2 ng, 4 ng, or 8 ng of Axin2 MO. For each injection, 10 embryos were de-chorionated and de-yolked at 30% epiboly prior to the addition of 100 µL Radio Immuno-Precipitation Assay (RIPA; 20mM Tris-HCl pH8.0, 140 mM NaCl, 10% glycerol, 2 mM EDTA, 1% NP-40, 0.1% Sodium Dodecyl Sulfate [SDS], 1% Sodium Deoxycholate, 1 mM phenylmethanesulfonylfluoride, 1 mM Sodium Orthovanadate, 10µg/mL Aprotinin, 10µg/mL Leupeptin) buffer. Samples were then stored on ice and 100 µL of 2x Loading Buffer (100 mM Tris-HCl pH6.8, 200 mM Dithiothreitol, 4% SDS, 0.2% Bromophenol Blue, 20% glycerol) added before being boiled at 100°C for 5 minutes. Samples were stored at -20°C until needed. 20 µL of each sample was resolved on a 7% SDS polyacrylamide gel via electrophoresis and transferred using an Electrophoretic Wet Transfer Cell (Bio-Rad Laboratories, Mississauga, ON, Canada) to a polyvinylidene fluoride membrane. Transfer buffer contained 0.25 M Tris Base and 1.92 M Glycine. Membranes were blocked in Blocking Solution (5% non-fat milk powder, 3% Bovine Serum Albumin [BSA] in phosphate-buffered saline with 0.1% Tween20 [PBST]) for one hour at room temperature followed by incubation with primary monoclonal anti-myc antibody (Vanderbilt University Antibody Core Facility) diluted 1:2000 in Blocking Solution containing 0.10% sodium azide overnight at 4°C. Following incubation, membranes were washed for 5 minutes 3X in PBST before being incubated with a horseradish peroixdase (HRP) conjugated goat anti-mouse secondary antibody (Cell signaling technologies, Pickering, ON, Canada; mAb#2276) at 1:10 000 in PBST with
5% non-fat milk powder for one hour at room temperature. Membranes were then washed for 15 minutes 3X in PBST before using Luminata Crescendo Western HRP Substrate (Millipore, Etobicoke, ON, Canada) for detection. Quantification of western blot for verification of MO efficacy was done with densitometry and Image J software.

Microinjection

Embryos were microinjected with a PV820 pneumatic PicoPump (Word Precision Instruments, Sarasita, FL, USA). mRNA was loaded into glass capillaries pulled into needles using a P-1000 micropipette puller (Sutter Instruments, Novato, CA, USA). To obtain 1 nL of mRNA released from each pulse of injection, a bolus of 0.6mm radius was required. This was determined using the following calculation:

\[ r = \frac{1}{2} d \]
\[ \text{Volume of sphere} = \frac{4}{3}\pi r^3 \]
\[ d = 0.12 \text{ mm or } r = 0.06 \text{ mm (measured from staged micrometer)} \]
\[ \therefore \frac{4}{3}\pi(0.06)^3 = 0.0009\text{mm}^3 \approx 1 \text{ nL} \]

Pulse length and amount of pressure were adjusted to achieve a droplet of 0.12mm in diameter in halocarbon oil (Sigma Aldrich, Oakville ON, Canada) as measured with a stage micrometer (Figure 6).

Embryos used for microinjection were collected from wildtype AB* mating pairs directly following fertilization, and placed in 3% agarose gels containing 1mm wells in which embryos could be positioned. 1 nL of mRNA was injected into the yolk directly below the cells at the one- to two-cell stage. Early in development, embryos display a
phenomena known as cytoplasmic streaming, which describes the movement of nutrients from the yolk (vegetal pole) to the cells (animal pole) (Kimmel et al. 1995). This streaming delivers the mRNA directly into the cells, generating a relatively even distribution of mRNA. Following injection, embryos were incubated at 28.5°C and fixed in 4% PFA at various developmental time points. Phenotypic analysis was conducted on embryos which had been microinjected at the one- to two-cell stage with either $axin2$ mRNA or $axin2$ MO and incubated at 28.5°C. Microinjections were done independently three times. In addition, 20 embryos of each microinjection type ($axin2$ or $axin2$ MO) were observed at 1 day post fertilization (dpf) resulting in a sample size of $n = 20$. 
Figure 6: Schematic representation of mRNA measurement. Diameter (0.12mm) of mRNA bolus (red) is being measured on staged micrometer slide to obtain a bolus of 1 nL.
**In Situ Hybridization**

Embryos were fixed at specific stages of development based on hpf and developmental stages according to Westerfield (2007). Staged embryos were fixed in one of two ways: wildtype embryos were either fixed directly in 4% PFA or were first microinjected with either mRNA or MO prior to fixation in 4% PFA. Following fixation overnight, embryos were washed in 1xPBST twice for 5 minutes and chorions were removed. Batches of 18-20 fixed and dechorionated embryos were then stored in methanol at -20°C until needed for *in situ* hybridization. *In situ* hybridizations were carried out either by hand, or using an in situ robot (InsituPro VSI, Intavis Bioanalytical Instruments, Cologne, Germany). While both methods used the same protocol, as described by Theisse et al. (1993), *in situ* hybridization experiments to determine the spatial and temporal expression of *axin2* and to determine if Wnt signaling is both sufficient and necessary to induce *axin2* expression were carried out by hand, while experiments to determine if *axin2* is necessary and sufficient to antagonize Wnt signaling were carried out using InsituPro VSI (Intavis Bioanalytical Instruments). All probes were applied to embryos at 1 µg/mL. By hand, embryos were hybridized at 70°C overnight, while in the robot at 68°C, as higher temperatures could not be reached. With *in situ* robot, alternate temperatures (55°C, 60°C, and 65°C) were attempted to increase intensity of staining, but without success. Following hybridization in both methods, embryos were stringently washed twice in 0.2x Sodium Chloride-Sodium Citrate Buffer (SSC) at 70°C for 30 minutes each, placed in block (PBST, 2% sheep serum, 2mg/mL BSA) for 3 hours at room temperature and incubated in anti Digoxigenin-Alkaline Phosphatase Fab Fragments (Roche) antibody diluted to 1:10 000 in block. Following
overnight detection, embryos were washed for 15 minutes 6x in PBST and washed three times in solution buffer containing 100mM TrisHCl pH 9.5, 50mM MgCl₂, 100mM NaCl₂ and 0.1% tween20 for 10 minutes each. Finally, embryos were removed and placed in 24-well plates, excess solution buffer removed and 400µL BM Purple AP Substrate (Roche) added to each well. Depending on the probe, staining was performed at room temperature for 2hrs – 3 days. Staining was stopped by washing embryos for 5 minutes 4X in Stop Solution (1XPBS, 0.2% Tween20, 50mM EDTA, 0.1% Sodium Azide, pH 5.5). For imaging, embryos were first rinsed for 10 minutes each in a graded series of 25% glycerol /75% Stop Solution, 50% glycerol/50% Stop Solution and 75% glycerol/25% Stop Solution. Once embryos were in 75% glycerol/25% Stop Solution, embryos were mounted on a depression slide for observation and imaged with a Zeiss Discover, V8 compound microscope (Zeiss, Toronto, Ontario, Canada) and MicroPublisher 5.0 camera (QImaging, Surry, BC, Canada). Embryos were stored in 75% glycerol/25% Stop Solution at 4°C in the dark.

**Measurement of the arc of gsc**

In overexpression and knockdown of axin2 expression experiments, gsc expression within the dorsal organizer was measured as an indicator of active Wnt signaling. Gsc expression resulted in a ventrolateral arc within the dorsal organizer of each embryo and was quantified using a dissecting microscope containing a 180° reticle eyepiece (Figure 7). Embryos were arranged in animal view with the center of each animal pole placed at the 90°/0° apex. Embryos were then arranged with arcs of staining
beginning at 0° and extending towards 180°, in order to measure the arc angle $\theta$. All measurements for each embryo were made at 50x magnification.
**Figure 7:** Arc of *goosecoid* (*gsc*) staining measurement. 180° reticle was placed in the eyepiece of a dissecting microscope and embryos arranged in animal view. The animal pole center of each embryo was placed at 90°/0° apex and the arc of staining aligned to extend from 0°- 180°.
CHAPTER 3: RESULTS & DISCUSSION
The spatial and temporal expression of \textit{axin2} during zebrafish development

Axin2 has recently been identified as a potential inducible negative regulator of Wnt signaling (Zeng \textit{et al.}, 1997; Jho \textit{et al.}, 2002; Leung \textit{et al.}, 2002; Yu \textit{et al.}, 2005). As a comprehensive study exploring the expression and function of Axin2 \textit{in vivo} has yet to be determined, I sought to first establish a spatial and temporal expression profile of Axin2 throughout zebrafish development in order to gain insight into its function. Additionally, I hope that by generating a comprehensive Axin2 expression profile I can address potential functional differences between Axin2 and Axin1.

\textit{Axin2 expression mimics Nkd1 expression in early zebrafish development}

To determine the spatial and temporal expression of \textit{axin2} in early development, embryos were harvested at dome (4.3hpf), 30\% epiboly (4.7hpf), 50\% epiboly (5.3hpf), 70\% epiboly (8hpf), and 6 somite (12hpf) stages for whole mount \textit{in situ} hybridization (Figure 8). Initially, multiple trials using \textit{Axin2} 5’UTR probe displayed no expression at any time points, while \textit{axin2} ORF probe displayed ubiquitous expression at all time points (data not shown). However, upon constructing an \textit{axin2} 3’UTR probe, unique domains of \textit{axin2} expression were detected within embryos. Expression of \textit{axin2} is ubiquitous at dome stage (Figure 8A, F). By 30\% epiboly, when Wnt signaling beings transitioning into zygotic control, expression becomes distinct within the ventrolateral border along the blastoderm margin (Figure 8B, G). This expression pattern seems to mimic that of the known Wnt signaling target, \textit{nkd1} (Van Raay \textit{et al.}, 2007). \textit{Axin2} expression continues to become more robust at 50\% epiboly, as expression expands
vertically towards the animal pole (Figure 8C, H), but is also lost within the presumptive dorsal organizer (Figure 8H). By 70% epiboly expression continues along the blastoderm margin, expanding the ventrolateral border of expression towards the vegetal pole (Figure 8D, I). Expression at this time is lost within the axial ectoderm and mesoderm creating a furrow of limited expression as indicated by the arrowhead (Figure 8D). Upon the initiation of segmentation, expression was observed within the tailbud and the developing somites (Figure 8E, J). Generally, axin2 expression was observed along the ventral lateral axis, in the mid-hindbrain (mhb) region, and developing somites throughout embryonic development. Axin2 3’UTR sense probe controls gave minimal signal indicating that hybridization was specific (Figure 9).
Figure 8: Expression of *axin2* during development using 3’UTR antisense probe. Lateral view (A-C, J), dorsal view (D, E) and animal view (F-I), of wildtype embryos at dome, 30% epiboly, 50% epiboly, 70% epiboly and 6 somite stages as shown. Arrowheads indicate axial mesoderm (D), dorsal organizer (H), tailbud (J) and midbrain hindbrain (mbh) (J). 30 embryos at each stage of development were fixed for *in situ* hybridization from the same hatchling of eggs resulting in a sample size of n = 30. Additionally, this was conducted twice, for a replicate number of n = 2.
Figure 9: View of embryos at dome and 50% epiboly following hybridization with *axin2* 3’UTR sense probe.
Discussion

I have reported a preliminary profile of axin2 expression in the early developing zebrafish embryo beginning at 4hpf and ending at 12hpf. Previous studies have shown that Axin2 can act as a negative regulator of Wnt signaling (Yan et al., 2001; Jho et al., 2002). Axin1 has also been demonstrated to down regulate Wnt signaling (Heisenberg et al., 2001), but is expressed ubiquitously throughout early development up until 12hpf, with expression increasing slightly towards the anterior of the embryo as development progresses into gastrulation (Kimmel et al., 1995; Heisenberg et al., 2001). Here I have found that zebrafish axin2 is also expressed ubiquitously at dome stage (4hpf), similar to axin1 (Figure 8A, F). This differs from known targets of maternal Wnt signaling, such as boz and gsc, which show restricted expression within the developing dorsal organizer (Figure 5A). However by 30% epiboly, only 45 minutes after dome stage, axin2 expression is no longer ubiquitous and becomes limited to ventrolateral domains of the embryo (4.7hpf) (Figure 8B, G). This shift from ubiquitous to ventrolateral expression presents a difference in axin1 and axin2 expression in early development and suggests the potential for differences in regulatory roles on Wnt signaling. Interestingly, as development continued to 50% epiboly ventrolateral expression remained (Figure 8C, H). This ventrolateral expression is similar to that of nkd1 (Van Raay et al., 2007). Nkd1 is a known universal target of Wnt signaling and an inducible regulator of the pathway. Nkd1 expression is robust along the medial blastoderm margin at 50% epiboly and showed expression in the developing somites and tailbud regions at segmentation stages (Van Raay et al., 2007). At 50% epiboly I also found axin2 was expressed along the ventrolateral marginal border as well as in somites and the tailbud region at the 6-somite
stage (Figure 8E, J). These similarities in axin2 and nkd1 expression support my hypothesis that axin2 is acting as an inducible regulator of the pathway. In spite of this, multiple points of difference between axin2 and nkd1 expression remain and must also be noted. For example, nkd1 expression was induced within the dorsal organizer at dome stage, while axin2 displayed ubiquitous expression (Figure 8A, F). Additionally, I also showed loss of axin2 expression within the presumptive axial margin at 70% epiboly (Figure 8D), while nkd1 expression seems to increase in axial mesoderm derivatives at this point in development (Van Raay et al., 2007). Axial mesoderm is responsible for the extension of the body axis and separation of the eye fields in the overlying neuroectoderm via shh signaling (Echelard et al., 1993). Lateral or non-axial mesendoderm however, is required for patterning ventral mesendoderm fates (Lekven et al., 2001) and for patterning the forebrain (Erter et al., 2001; Lekven et al., 2001). Thus, the complimentary expression patterns of nkd1 and axin2 suggest that while axin2 and nkd1 may share regulatory roles on Wnt signaling, expression in different tissues may be required for proper neuroectoderm, forebrain and eye formation. Taken together, a comparison of nkd1 and axin2 expression at varying developmental time-points could provide insight into how they regulate Wnt signaling to ensure proper patterning and axis formation.

Perhaps the most striking observation in our analysis of axin2 expression is that its expression coincides with known regions of active Wnt signaling throughout early zebrafish development. For example, ventrolateral expression observed at 30% and 50% epiboly is congruent to known domains of active Wnt signaling (Dorsky et al., 2002). In
Dorsky et al. designed an *in vivo* reporter system (TOPdGFP) to examine targets of β-catenin induced Wnt signaling by identifying β-catenin responsive cells during development. Under the control of a β-catenin driven promoter, active Wnt signaling expression was detected in the ventrolateral mesendoderm, which has now been identified as the earliest site of zygotic Wnt activity by Wnt8. From 30% - 50% epiboly, I also observed expression of *axin2* in the ventrolateral border of the blastoderm (Figure 8B-C, G-H), which recapitulates ventrolateral expression of Wnt8 seen in the TOPdGFP reporter line (Dorskey et al., 2002; Ramel and Lekven, 2004). Additionally, zygotic ventrolateral Wnt8 expression restricts the expansion of dorsal organizing genes which are being upregulated by maternal Wnt signaling (Erter et al., 2001). Therefore, more robust expression of *axin2* within ventrolateral mesoderm at 50% epiboly (Figure 8C), as well as loss of *axin2* expression within the presumptive dorsal organizer along the dorsal midline (Figure 8H), demonstrates *axin2* expression coincides with these established active and non-active regions of Wnt signaling. Active domains of Wnt signaling have also been identified in the mbh boundary and tailbud regions (Figure 8J). In particular to the tail bud region, reporter line β-catenin expression was highest in presomitic mesoderm and the newest somites (Dorsky et al, 2002). Within the developing head, this same reporter line displayed strongest expression in the mbh boundary and eyes, which coincides with Wnt1 and Wnt10b expression (Dorsky et al., 2002). As such, both tail bud and mbh domains display active Wnt signaling identified in earlier studies and with the TOPdGFP reporter line, and were analogous to *axin2* expression observed here at the 6 somite stage (Figure 8J). The establishment of overlap between known regions of active Wnt signaling and *axin2* expression is crucial in our investigation of *axin2* in Wnt
signaling, as overlapping domains would be a prerequisite for axin2 to act as a negative feedback regulator of Wnt-mediated signaling. Additionally, the strikingly similar expression profile to the known inducible inhibitor nkd1, as well as differences in expression between axin2 and the constitutively active Wnt signaling inhibitor axin1, further build on the idea that axin2 is acting as an inducible regulator.
Wnt signaling is both sufficient and necessary to induce \textit{axin2} expression

Having established that the expression of \textit{axin2} in early zebrafish development coincides with known domains of active Wnt signaling, I next wanted to determine whether Wnt signaling was both sufficient and necessary to induce \textit{axin2} expression. Since Van Raay \textit{et al.} (2007) established that \textit{nkd1} was an obligate and inducible target of canonical Wnt signaling, I compared \textit{axin2} to \textit{nkd1} expression following experimental activation of the canonical Wnt signaling pathway.

1. \textit{Wnt Signaling is sufficient to induce \textit{axin2} expression}

To confirm that \textit{nkd1} is a target of Wnt signaling, embryos were microinjected at the one- to two-cell stage with constitutively active βcatenin (ΔNβcatenin) to induce expression of Wnt responsive target genes. These embryos were then assayed for ectopic expression of \textit{nkd1} using an antisense \textit{nkd1} ORF probe. At both 30\% and 50\% epiboly, uninjected embryos exhibit a distinct pattern of \textit{nkd1} ventrolateral expression, which is consistent with known domains of active Wnt signaling (Figure 10A, C) (Dorskey \textit{et al.}, 2002; Van Raay \textit{et al.}, 2007). Following microinjection of 50 pg ΔNβcatenin, ectopic expression of \textit{nkd1} was observed in clusters of cells within the animal cap, which confirms that Wnt signaling is sufficient to induce \textit{nkd1} expression (Figure 10B, D).

Next, to determine whether \textit{axin2} expression mimics that of \textit{nkd1}, 20 embryos of each experimental type (uninjected 30\% epiboly, uninjected 50\% epiboly, ΔNβcatenin 30\% epiboly, and ΔNβcatenin 50\%epiboly) were harvested from the same set of
uninjected and ΔNβcatenin microinjected embryos used for the investigation of *nkd1* expression; however, embryos were instead assayed for *axin2* expression using an *axin2* 3’UTR probe. At both 30% and 50% epiboly, uninjected embryos display the same ventrolateral expression of *axin2* as seen with *nkd1* expression, but with less intensity (Figure 11A, C). Following microinjection with 50 pg ΔNβcatenin, embryos also display ectopic *axin2* expression in clusters within the animal cap (Figure 11B, C), which mimics that seen in *nkd1* experiments (Figure 10B, D). Wildtype and microinjected embryos at 30% and 50% epiboly were also exposed to sense *axin2* 3’UTR probe as a negative control, and all embryos remained colourless (data not shown). Therefore, I conclude that Wnt signaling is sufficient to induce *axin2* expression.
Figure 10: Wnt signaling induces *nkd1* expression. Embryos are arranged in animal view at 30% (A, B) and 50% (C, D) epiboly displaying *nkd1* expression. (B, D) Embryos at both 30% (n=38, pooled from 2 independent sets of microinjection) and 50% (n=42, pooled from 2 independent sets of microinjection) epiboly following ΔNβCatennin microinjection display ectopic expression (darkened regions) in the animal cap relative to uninjected embryos (A, C).
Figure 11: Wnt signaling induces *axin2* expression. Embryos are arranged in animal view at 30% (A, B) and 50% (C, D) epiboly displaying *axin2* expression. (B, D) At both 30% (n=39, pooled from 2 independent sets of microinjection) and 50% (n=42, pooled from 2 independent sets of microinjection) epiboly, ΔN\(\beta\)Catenin microinjected embryos display ectopic expression (darkened regions) in the animal cap relative to uninjected embryos (A, C) which display only ventrolateral *axin2* expression.
2. *Wnt Signaling is necessary to induce axin2 expression*

As Wnt signaling is sufficient to induce *axin2* expression, I next wanted to investigate whether Wnt signaling was necessary for *axin2* induction. I first wanted to re-establish whether Wnt signaling was necessary to induce *nkd1* expression (Van Raay *et al.*, 2007). To accomplish this, I overexpressed Axin1. Axin1 has been identified as a constitutive inhibitor of Wnt signaling throughout development (Heisenberg *et al.*, 2001). Knockdown or mutant Axin1 results in reduced or absent eyes and reduced telencephalon development, indicative of posterior neural fate development at the expense of anterior tissues, and is consistent with overactive Wnt signaling (Heisenberg *et al.*, 2001; van de Water *et al.*, 2001). Conversely, overexpression of Axin1 results in an enlarged forebrain, indicative of reduced Wnt signaling (Heisenberg *et al.*, 2001). With this knowledge, I microinjected embryos at the one- to two-cell stage with 200 pg *axin1* to knockdown Wnt signaling and assayed for *nkd1* or *axin2* expression. Injected and uninjected embryos were harvested at both 30% and 50% epiboly and both treatments developed for the same period of time. At both 30% and 50% epiboly, uninjected embryos again displayed ventrolateral *nkd1* expression, consistent with our previous results (Figure 12A, C). In contrast, embryos overexpressing Axin1 displayed significantly less ventrolateral expression of *nkd1* with limited expression in the presumptive dorsal organizer (Figure 12B, D). These results confirm previous findings that Wnt signaling is necessary to induce *nkd1* expression and identify *axin2* as a novel target of Wnt signaling.
Having established that Wnt signaling is necessary to induce *nkd1* expression, I next investigated if Wnt signaling was necessary to induce *axin2* expression. Following microinjection with 200 pg *axin1* to knockdown Wnt signaling, loss of *axin2* expression was almost identical to *nkd1* expression following microinjection. Using the *axin2* 3’UTR probe, at 30% epiboly embryos show almost complete loss of *axin2* expression relative to uninjected embryos, with very subtle hints of expression within the putative dorsal organizer (Figure 13B). By 50% epiboly, *axin2* expression is completely lost (Figure 13D). Uninjected and microinjected embryos at 30% and 50% epiboly were also exposed to sense *axin2* 3’UTR probe as a negative control, and all embryos displayed no expression (data not shown). Therefore, I conclude that Wnt signaling is necessary to induce *axin2* expression.
Figure 12: Wnt signaling is necessary to induce *nkd1* expression. Embryos are arranged in animal view at 30% (A, B) and 50% (C, D) epiboly. (B, D) At both 30% (n=38, pooled from 2 independent sets of microinjection) and 50% (n=40, pooled from 2 independent sets of microinjection) epiboly, Axin1 microinjected embryos display reduced *nkd1* expression relative to uninjected embryos (A, C).
Figure 13: Wnt signaling is necessary for axin2 expression. Embryos are arranged in animal view at 30% (A, B) and 50% (C, D) epiboly displaying loss of axin2. (B, D) At both 30% (n=34, pooled from 2 independent sets of microinjection) and 50% (n=39, pooled from 2 independent sets of microinjection) epiboly, axin1 microinjected embryos display reduced axin2 expression relative to uninjected embryos (A, C).
Discussion

These results demonstrate that canonical Wnt signaling is both sufficient and necessary to activate axin2 expression. Activation of Wnt signaling through overexpression of constitutively active β-catenin, induced ectopic expression of both nkd1 and axin2 in the animal cap (Figure 10B, D; Figure 11B, D). The animal cap cells at 30% and 50% epiboly are not expected to have Wnt signaling according to a Wnt reporter line (Dorskey et al., 2002). Thus, nkd1 and axin2 expression in these cells following synthetic Wnt signaling induction indicates Wnt signaling is sufficient to induce nkd1 and axin2 expression and confirms the results with nkd1, which have previously been demonstrated (Van Raay et al., 2007). Additionally, following microinjection with axin1 mRNA, nkd1 and axin2 expression was abolished leaving only minimal amounts of expression within the putative dorsal organizer (Figure 12B, D; Figure 13B, D). Reduction of nkd1 and axin2 expression following knockdown of Wnt signaling indicates Wnt signaling is necessary to induce nkd1 and axin2 expression. While I have demonstrated that Wnt signaling is sufficient and necessary to induce nkd1 and axin2 expression, this can be strengthened with additional investigation. In particular, the use of mutants which display perturbed Wnt signaling would greatly reinforce our findings of how Wnt signaling is acting on axin2 expression. Between the stages of dome to 30% epiboly, Bozozok (boz) is a direct target of maternal canonical Wnt signaling (Kawahara et al., 2000; Bellipanni et al., 2006) and is further required for normal dorsal organizer development and subsequent expression of dorsal organizer markers such as gsc and chd (Miller-Bertoglio et al., 1997). These dorsal organizer genes are important for proper dorsal structure (such as the brain and spinal cord)
development, axis formation, and neuroectoderm specification (Saude et al., 2000; Lyman Gingerich et al., 2005; Dixon Fox and Bruce, 2009). Boz mutants display defects in the development of axial structures due to expansion of Wnt8 into the dorsal blastoderm (Fekany et al., 1999). Therefore, boz is required for the induction of dorsal organizing genes, which subsequently restricts mediolateral Wnt8 expansion within the dorsal blastoderm (Fekany et al., 1999; Solnica-Krezel and Driever, 2001). As axin2 is a target of Wnt signaling, expansion of axin2 into the dorsal blastoderm margin would be expected in boz mutants at 50% epiboly (Figure 8H arrowhead). If observed, this would provide evidence in a physiologically relevant system that confirms my observation that Wnt signaling is sufficient to induce axin2 expression.

Evidence remains, however, that axin2 may not be solely restricted to Wnt signaling. As seen previously, axin2 expression was ubiquitous at dome stage (Figure 8A, F) and not restricted to the developing dorsal organizer, suggesting axin2 expression may be regulated by other signaling pathways. It has been identified that Nodal signaling is active within marginal cells during late blastula (30% epiboly) and early gastrula (50% epiboly) stages and is crucial in directing germ layer formation, the establishment of the dorsal organizer, and the organization of the anterior-posterior axis (Gritsman et al., 1999; Shimizu et al., 2000; Bennett et al., 2007). Additionally, active BMP signaling and Nodal specific gene expression is also ubiquitous at dome stage (Lim et al., 2000; Bennett et al., 2007), suggesting potential regulation and activation of axin2 expression by additional signaling pathways. Therefore, further experiments are needed to verify whether axin2 is a unique target of Wnt signaling in zebrafish, or whether pathways such
as BMP and Nodal also regulate its expression. Nonetheless, these experiments establish

*axin2* as a target of Wnt signaling, but whether it is a direct and restricted target of Wnt signaling requires further experimentation.
Axin2 is sufficient and necessary to antagonize Wnt signaling

I next wanted to focus our investigation on the role of axin2 and how it influences Wnt signaling dynamics. Previous studies have demonstrated that Axin1 is an important regulator of Wnt signaling (Heisenberg et al., 2001; Stoick-Cooper et al., 2007) and that its parologue axin2 can recue axin1−/− embryonic lethality (Chia and Costantini, 2005). In addition, it has been observed that axin2 is capable of decreasing levels of β-catenin, which subsequently inhibits the expression of Wnt target genes (Behrens et al., 1998; Furuhashi et al., 2001; Chia and Costantini, 2005). To verify and build on previous findings suggesting that axin2 is sufficient to antagonize Wnt signaling in zebrafish, I sought to determine whether axin2 is not only sufficient but also necessary to antagonize Wnt signaling using both knockdown and overexpression experiments.

1. Overexpression and knockdown of Axin2 affects zebrafish development

I first wanted to determine if the overexpression or knockdown of axin2 would have gross morphological effects on zebrafish development. Microinjection of synthetic axin2 mRNA or axin2 MO was performed on embryos at the one- to two-cell stage to induce overexpression or knockdown of axin2 respectively. Initial investigation indicated that both conditions resulted in similar phenotypes with shortened axis, including reduced somite formation, and defects in head and tail formation (Figure 14B, C). However, upon closer examination there were significant differences. Overexpression of zebrafish axin2 results in enlarged heads compared to uninjected siblings (Figure 14B). These embryos also display limited somite formation and perturbed extension movements resulting in an anterior-posterior shortening (Figure
14B). The large head and short tail are consistent with dorsalization of embryos upon overexpression of axin2. Additionally, an enlarged forebrain suggests the neuroectoderm has been anteriorized. I also demonstrate that knockdown of axin2 expression via axin2 MO microinjection results in embryos with shortened tails similar to their axin2 injected counterparts (Figure 14C). However, these embryos also displayed considerable necrosis and reduced cell proliferation within forebrains, including eyes (Figure 14C). These findings are consistent with Wnt8 overexpression studies in which embryos are ventralized and the neuroectoderm posteriorized, including the loss of eye development (Lekvin et al., 2001).
**Figure 14**: Overexpression and knockdown of *axin2* causes phenotypic variation at 1dpf. (A) Uninjected embryo. (B) Embryo at 1dpf following *axin2* overexpression displaying a shortened body plan and enlarged forebrain (n=89, from four independent rounds of microinjection) (C) Embryo at 1dpf following Axin2MO knockdown displaying similar shortened body plan, necrosis and loss of eyes (n=78, from four independent rounds of microinjection).
2. *Axin2 is sufficient to antagonize Wnt signaling*

*i. Axin2 can reduce gsc and chd expression*

The phenotypes observed in Figure 14 at 1dpf could be due to effects on several different Wnt signaling events during the first 24 hours of development. To further understand and clarify the action of *axin2*, I chose to look at effects earlier in development, during known Wnt signaling events. To accomplish this, I injected synthetic *axin2* RNA into embryos at the one- to two-cell stage and assayed embryos at dome, 30% epiboly, and 50% epiboly for *gsc* and *chd* expression within the dorsal organizer. *Gsc* and *chd* have both been well characterized as dorsal organizer markers that are responsive to Wnt signaling (Miller-Bertoglio *et al.*, 1997; Schulte-Merker *et al.*, 1994; Saude *et al.*, 2000). Injection of *axin2* resulted in complete loss of *chd* expression within the dorsal organizer at dome stage (Figure 15B). By 30% and 50% epiboly, marked reductions in *chd* expression within the organizer were observed (Figure 15D, F). In agreement with *chd* expression data, we also observed a reduction in *gsc* expression following injection of *axin2* mRNA (Figure 16). At dome stage, uninjected and *axin2* injected embryos displayed very low levels of expression within the dorsal organizer (Figure 16A, B). However by 30% epiboly, mediolateral expression of *gsc* was almost completely abolished in *axin2* microinjected embryos relative to uninjected controls (Figure 16C, D). By 50% epiboly, reduction in *gsc* expression remained but the degree of suppression was reduced, as embryos displayed limited amounts of *gsc* expression within the dorsal organizer (Figure 16F).
Chd expression fluctuates both ventrolaterally and along the animal/vegetal axis, while gsc expression is very defined ventrolaterally. Therefore, chd expression was not measured, while the angle created by the defined ventrolateral arc of gsc expression was measured with a 180° reticle and light microscope (Figure 17). Embryos measured were pooled (n1) from several independent trials (n2). The mean arc of gsc expression in uninjected embryos from four independent trials at 30% epiboly was 22.7° (n1 = 63, n2 = 4, SE = ± 0.6°) and 1.95° following axin2 overexpression (n1 = 62, n2 = 4, SE = ± 0.3°). At 50% epiboly, the mean arc of gsc expression was 14.8° in uninjected embryos (n1 = 61, n2 = 4, SE = ± 0.5°) and 2.6° in embryos following axin2 microinjection (n= 58, n2 = 4, SE = ± 0.3°). A two-way ANOVA was conducted with two factors: treatment (axin2 microinjected or uninjected) and stage of embryo collection (30% or 50% epiboly). With these factors, a two-way ANOVA examined the effect of each factor on the arc of gsc expression. Treatment type and stage at which embryos were collected had a significant effect on the amount of gsc expression (F_{2,3} = 141.4, p < 0.05; F_{2,3} = 17.1, p = 0.001). In addition, the interaction between treatment and stage of embryo collection was significant (F_{2,3} = 5.6, p = 0.013). Bonferroni post-hoc results further indicated that at both 30% epiboly (mean = -20.7° ± 2.5, p < 0.05) and at 50% epiboly (mean = -12.2° ± 1.5, p < 0.05) axin2 significantly reduced the mean arc of gsc expression by 20.7° and 12.2° respectively (Figure 17).
Figure 15: Axin2 inhibits chd expression. Embryos arranged in animal view at dome (A, B), 30% epiboly (C, D) and 50% epiboly (E, F) displaying chd expression following an Axin2 microinjection. (B, D) Embryos at dome (n=59, from four independent rounds of microinjection) and 30% (n=62, from four independent rounds of microinjection) epiboly following axin2 microinjection display marked reduction in chd expression relative to uninjected embryos (A, C), which display expression within the dorsal organizer. (F) Reduction in chd expression is also observed at 50% epiboly (n=60, from four independent rounds of microinjection).
Figure 16: *Axin2* inhibits *gsc* expression. Embryos arranged in animal view at dome (A, B), 30% epiboly (C, D) and 50% epiboly (E, F) displaying *gsc* expression following an *Axin2* microinjection. (A, B) Uninjected and *axin2* injected embryos (n=64, from four independent rounds of microinjection) display limited expression within the dorsal organizer at dome stage. (D) Embryos at 30% epiboly following *axin2* microinjection (n=61, from four independent rounds of microinjection) display marked reduction in *gsc* expression relative to uninjected embryos (C) which display expression within the dorsal organizer. (F) As development progresses from 30% to 50% epiboly, reduction in *gsc* expression is observed, however some expression is regained within the dorsal organizer.
Figure 17: Mean arc of gsc expression at 30% and 50% epiboly in uninjected and axin2 microinjected embryos. Error bars represent standard error (Bonferroni post-hoc, *, p<0.05).
3. Axin2 is necessary to antagonize Wnt signaling

i. Loss of axin2 expands gsc and chd expression

To examine whether axin2 was necessary to antagonize Wnt signaling, I designed complementary, antisense MO to target the ATG transcriptional start site of axin2 so as to repress translation of maternal and zygotic axin2 RNA. To determine the optimal concentration of axin2 MO, serial increases of axin2 MO were injected into embryos and phenotypes observed. Microinjection of 4-8 ng of axin2 MO resulted in 50-90% embryo death by 6 hpf with the remaining embryos suffering from necrosis and severe developmental defects relative to WT embryos (data not shown). Microinjection of 2ng Axin2 MO resulted < 20% embryo death along with minimal and non-specific necrosis until 1 dpf, therefore unless otherwise noted, 2ng of Axin2 MO was used during microinjection. To determine the efficacy of MO, I co-injected MO along with myc-Axin2 and assayed by Western blot analysis the effect of increased amounts of MO on Axin2-myc protein levels. Embryos were first injected with equal amounts of Axin2-myc (200ng/µL) followed by a co-injection of either 0ng, 2ng, 4ng or 8ng of axin2 MO. Western blot followed by densitometry analysis indicated serial decreases in Axin2-myc protein levels as axin2 MO concentration increased (Figure 18). Densitometry quantification was conducted with Image Lab software. These results confirm axin2 MO is capable of inhibiting axin2 expression.

Injection of axin2 MO at the one- to two-cell stage resulted in a modest increase of chd expression along the anterior-posterior axis at dome stage (Figure 19B), while expansion in expression continued to broaden along the mediolateral border as
development progressed to 30% epiboly (Figure 19D). By 50% epiboly chd expression had consistently expanded along both lateral and longitudinal axes (Figure 19F). Consistent with chd expression, gsc expression also expanded following axin2 MO injection at all three stages of development, but expansion was more limited mediolaterally compared to uninjected embryos (Figure 20).

To quantify expansion along the mediolateral border, the arc of gsc expression was again measured. Embryos measured were pooled \( (n_1) \) from several independent trials \( (n_2) \). Mean arc of gsc expression in axin2 MO microinjected embryos at 30% epiboly was 28.11° \((n = 62, n_2 = 4, \text{SE} = \pm 0.8°)\) and 22.7° in uninjected siblings \((n_1 = 63, n_2 = 4, \text{SE} = \pm 0.6°)\). At 50% epiboly, the mean arc of gsc expression was 14.8° in uninjected embryos \((n = 61, n_2 = 4, \text{SE} = \pm 0.5°)\) and 21.32° \((n = 63, n_2 = 4, \text{SE} = \pm 0.7°)\) in embryos following axin2 MO microinjection (Figure 21). A two-way ANOVA was conducted using two factors: treatment \((\text{axin2 MO microinjected or uninjected})\) and stage of embryo collection \((30\% \text{ or } 50\% \text{ epiboly})\). With these factors, a two-way ANOVA examined the effect of each factor on the arc of gsc expression. Treatment type and stage at which embryos were collected had a significant effect on the arc of gsc expression \((F_{2,3} = 141.4, p \leq 0.05; F_{2,3} = 17.1, p = 0.001)\). Additionally, the interaction between treatment and stage of embryo collection was significant \((F_{2,3} = 5.6, p = 0.013)\). Bonferroni post-hoc results further indicated at 50% epiboly axin2 MO significantly expanded the mean arc of gsc expression by 6.5° \((\text{mean} = 6.5° \pm 1.3, p = 0.002)\) (Figure 21). Interestingly and in contrast to axin2 overexpression, axin2 MO injection did not significantly affect gsc expression at 30% epiboly (Figure 21).
**Figure 18:** Verification of *axin2* MO efficacy. (A) Following co-injection with 2-8ng of *axin2* MO, western blot analysis of 10 embryos (pooled) revealed a decrease in Axin2 expression as MO concentration increased. Bands correspond to Axin2 in the upper panel and actin, used as a loading control. (B) Densitometry quantification of Axin2 bands was conducted using Image Lab software and is plotted after normalization to Actin in the same sample.
**Figure 19:** Knockdown of *axin2* results in expanded *chd* expression. Embryos are arranged in animal view at dome (A, B), 30% epiboly (C, D) and 50% epiboly (E, F) displaying *chd* expression following *axin2* MO microinjection. (B) Embryos at dome stage following *axin2* MO microinjection (n=59, from four independent rounds of microinjection) display a moderate increase in *chd* expression. (D) At 30% epiboly (n=63, from four independent rounds of microinjection), expansion in *chd* expression relative to uninjected embryos (C) continued to broaden mediolaterally within the dorsal organizer. (F) By 50% epiboly (n=60, from four independent rounds of microinjection) expansion in *chd* expression began to increase along the anterior-posterior axis to broaden breadth of expression within the dorsal organizer.
Figure 20: Knockdown of *axin2* results in expanded *gsc* expression. Embryos are arranged in animal view at dome (A, B), 30% epiboly (C, D) and 50% epiboly (E, F) displaying *gsc* expression following *axin2* MO microinjection. (B) Embryos at dome stage following *axin2* MO microinjection (n=61, from four independent rounds of microinjection) display a moderate increase in *gsc* expression relative to uninjected controls (A). (D, F) At 30% (n=62, from four independent rounds of microinjection) and 50% (n=59, from four independent rounds of microinjection) epiboly, expansion in *gsc* expression relative to uninjected embryos (C, E) continued to broaden mediolaterally within the dorsal organizer.
Figure 21: A graph of mean arc of gsc expression at 30% and 50% epiboly in uninjected embryos and axin2 MO microinjected embryos. Error bars represent standard error (Bonferroni post-hoc, *, p = 0.02).


Discussion

In determining whether axin2 can antagonize Wnt signaling, I first examined whether overexpression or knockdown of axin2 would induce gross phenotypic affects in embryos at 1dpf. Genetic studies have shown that Wnt8 signaling is essential in the establishment of posterior neural fates, responsible for the establishment and positioning of the midbrain-hindbrain boundary and involved in ventral-lateral mesodermal patterning (Erter et al., 2001; Lekven et al., 2001; Rhinn et al., 2005;). In addition, studies conducted by Erter et al. (2001), which knockdown wnt8 expression via wnt8 MO microinjection, resulted in embryos at 1dpf exhibiting expanded anterior neural fates at the expense of posterior neuroectoderm. In my results, following the overexpression of axin2 to inhibit Wnt signaling, I observed enlarged forebrains and loss of the midbrain-hindbrain boundary (Figure 14B), which is consistent with the wnt8 knockdown phenotype. Furthermore, knockdown of axin2 expression via axin2 MO displayed loss or reduction of eye formation (Figure 14C), indicating a loss of anterior forebrain. As mentioned previously, Wnt8 is responsible for the establishment of posterior fates, with overexpression of wnt8 resulting in embryos with a headless-like phenotype (Lekven et al. 2001). Together, initial phenotypic analysis displaying opposing phenotypic characteristics following overexpression and knockdown of axin2 that mimic knockdown and overexpression of wnt8, respectively, suggests axin2 is acting as an antagonist of Wnt signaling in early development.

Defects in trunk and tail formation were also observed following axin2 overexpression and axin2 MO knockdown resulting in a reduction in somite formation.
Vertebrate trunk and tail formation begins as somites form on each side of the body axis during early development. Somites are patterned periodically, being laid down sequentially in a ‘clock-like’ process as presomitic mesoderm elongates in the tail of the embryo (Conlon et al., 1995; van Eeden et al., 1996; Aulehla et al., 2003). Cells within the presomitic mesoderm show an oscillating or cyclically timed expression pattern in zebrafish. As a result, segmentation occurs in a manner which seems to periodically progress from anterior to posterior presomitic mesoderm resulting in visible somites (Conlon et al., 1995; van Eeden et al., 1996; Aulehla et al., 2003). This oscillating, or timed expression pattern is referred to as a segmentation clock, with one clock cycle resulting in a single somite being formed on each side of the notochord. Of particular importance here is the identification of active Wnt signaling within segmentation and somite formation (Aulehla et al., 2003; Ozbudak and Lewis 2008). Interestingly, it has been proposed that a negative-feedback loop between Wnt3a and axin2 may play a role in clock and gradient formation to control the segmentation process (Aulehla et al., 2003). This is consistent with my observation of axin2 within the tail bud region (Figure 8J) and further explains why overexpression and knockdown of axin2 both resulted in perturbed posterior extension (Figure 14B, C). In both instances, each condition can interrupt the cyclical oscillation of stringently timed Wnt signaling events, effectively disrupting somite formation. Additionally, loss of Wnt8 expression and overexpression of Wnt antagonist Dkk1 have also resulted in embryos deficient in trunk and tail production as well, with only three or four poorly structured somites forming, further indicating the importance of properly timed expression and inhibition of Wnt signaling in axis elongation (Hashimoto et al., 2000; Shinya et al., 2000; Lekven et al., 2001).
While phenotypic analysis at 1dpf proved helpful in gaining initial insight into the regulatory function of *axin2* more detailed analysis of specific Wnt signaling events were required to further characterize Axin2 function. To accurately determine whether *axin2* is sufficient and necessary to antagonize Wnt signaling I examined known targets of Wnt signaling (*gsc* and *chd*) for expansions or reductions in expression following *axin2* overexpression or knockdown (Schulte-Merker et al., 1994; Miller-Bertoglio et al., 1997; Saude et al., 2000). I observed that overexpression of *axin2* caused a reduction in *chd* and *gsc* expression in the dorsal organizer at both 30% and 50% epiboly (Figure 15, Figure 16). In particular, I found overexpression of *axin2* had a significant effect on the arc of *gsc* expression, at both 30% and 50% epiboly (Figure 16D, F). Interestingly, while *gsc* expression was significantly reduced at both developmental time points, it is clear that some expression within the putative organizer remained or was re-gained as development progressed from 30% to 50% epiboly (Figure 16D, F). This may be explained by opposing maternal and zygotic Wnt signaling events occurring at 50% epiboly. At 30% epiboly, maternal Wnt signaling is inducing *boz* expression within the dorsal organizer, which in turn induces *gsc* expression (Dixon Fox and Bruce, 2009); however, by 50% epiboly zygotic induction of Wnt8 has commenced ventrolaterally, subsequently reducing the lateral expansion of *gsc* within the organizer (Figure 22B) (Dixon Fox and Bruce, 2009). Therefore, following *axin2* overexpression, we see a reduction in *gsc* expression at 30% epiboly consistent with Wnt signaling inhibition within the organizer. However at 50% epiboly when faced with two Wnt signaling events, I believe Axin2 is inhibiting ventrolateral Wnt8 signaling, allowing for the
repression of dorsal organizer Wnt signaling to be relieved and thus gsc expression to be re-gained (Figure 22B).

Conversely, we found that reducing axin2 expression through axin2 MO injection resulted in expansions of chd and gsc expression (Figure 19, Figure 20). With respect to gsc expression, significant increases in the arc of gsc expression were only found at dome and 50% epiboly, but not 30% epiboly (Figure 20). This non-significant result may be explained by the use of MO to knockdown axin2 expression, and opposing maternal and zygotic Wnt signaling events occurring between 30% - 50% epiboly. MOs are synthetically designed and require no modification once microinjected before exerting its effect. Therefore upon microinjection, axin2 MO is capable of initiating its function instantly and can act immediately on maternal mRNA. This is reflected in expression of gsc in treated embryos at dome stage relative to no expression in uninjected siblings (Figure 20A, B). As development continues to 30% epiboly, uninjected embryos must maintain a balance between dorsal and ventro-lateral expression, which is kept in check by maternal and zygotic Wnt signaling, respectively. As these two Wnt signaling events are temporally regulated, I would expect their sensitivity to either axin2 overexpression or knockdown to likewise be different at different developmental stages. Also, while MO’s act immediately, mRNA needs to be translated into protein and sufficient levels need to accumulate to exert an observable effect. Thus, due to timing of maternal vs. zygotic Wnt signaling events and the nature of how MOs knock down expression, I would not necessarily expect axin2 overexpression or knockdown to have opposite effects at early stages of development. For example, at 30% epiboly I observed no
significant expansion in arc of gsc expression despite axin2 MO microinjection (Figure 20C, D). This may be due to the fact that at this developmental stage, the primary maternally induced Wnt signaling event within the dorsal organizer is beginning to encounter the second opposing zygotic Wnt signaling event inhibiting dorsal organizer expansion ventrolaterally (Figure 22C). Additionally, at this particular concentration of MO microinjection, axin2 MO could be acting equally on both signaling events occurring at this time, in effect cancelling each other out, resulting in a non-significant result. Nevertheless, it was apparent that there was a slight increase in the arc of gsc expression at 30% epiboly, despite not being statistically significant, which is congruent with findings at early and later stages of development. By 50% epiboly, ventrolateral Wnt signaling begins to restrict expansion of the dorsal organizer ventrolaterally. However for 5.5 hours, or up until 50% epiboly, expression of dorsal organizer specific genes has expanded in treated embryos due to the effect of the axin2 MO microinjection at the one cell stage. Therefore upon initiation of Wnt8 signaling, expanded dorsal organizers are restricted from expanding further (Figure 22C). Taken together, I interpret these results to mean axin2 plays a sensitive role in regulating Wnt signaling in both dorsal organizer and ventrolateral domains. However, to truly elucidate axin2 repression of Wnt signaling at various developmental time points, it would be beneficial to observe nkd1 expression following axin2 MO injection, as it has been characterized as a target of Wnt signaling at these specific points in development (Van Raay et al., 2007). My phenotypic analysis as well as overexpression and knockdown supports the notion that Wnt signaling plays a central role in normal zebrafish development and suggests axin2 is sufficient and necessary to limit Wnt signaling. The inhibition of gsc and chd expression following
axin2 microinjection and subsequent loss of this repression following axin2 MO injection supports my hypothesis that axin2 is a negative regulator of the pathway. Collectively these results provide a better understanding of how Wnt signaling is regulated and helps define when it is active and inactive, as ultimately it is the precise regulation of Wnt activity that is crucial for normal development.
Figure 22: Summary of proposed regulation by axin2 of Wnt signaling. (A) gsc expression within the dorsal organizer is initiated at 30% epiboly as a result of maternally induced Wnt signaling. By 50% epiboly, induction of zygotic Wnt signaling restricts mediolateral expansion of gsc expression within the organizer, creating a scenario of two opposing Wnt signaling events. (B) Inhibiting maternal Wnt signaling eliminates gsc expression in the organizer at 30% epiboly, however at 50% epiboly inhibition of zygotic Wnt signaling allows gsc expression to be regained within the dorsal organizer. (C) Reducing axin2 function expands gsc expression within the dorsal organizer.
CHAPTER 4: SUMMARY AND CONCLUSIONS
In summary, investigation into the potential role of *axin2* in early zebrafish development has revealed that *axin2* plays an inducible role in Wnt signaling regulation. I have conducted a preliminary expression analysis of *axin2* during early zebrafish development and demonstrated that *axin2* expression closely mimics the expression of *nkd1*, a known inducible negative regulator of Wnt signaling, and overlaps with well characterized regions of active Wnt signaling within the developing embryo (Erter et al., 2001; Lekven et al., 2001; Van Raay et al., 2007). During this analysis I also describe differences in domains of *nkd1* and *axin2* expression, specifically as development continues beyond dome stage. Differential expression profiles between *nkd1* and *axin2* provide a potential starting point for future studies in determining the independent role each plays in guiding fate choices throughout development. Additionally, we show *axin2* can negatively regulate Wnt signaling and that its expression is Wnt induced. This builds on differences in *axin1* and *axin2* expression in early zebrafish development by providing insight into the importance of inducible and constitutive inhibition on Wnt signaling as they work together to dictate normal patterning throughout development. Taken together, the following a model of *axin2* inhibition of Wnt signaling can be proposed based on a summary of our analysis (Figure 22). *Axin2* overexpression and knockdown can abolish or expand, respectively, expression of known Wnt signaling target genes, such as *gsc* and *chd*, at dome, 30% and 50% epiboly indicating *axin2* is sufficient and necessary at antagonizing Wnt signaling.

In future experiments, it would prove interesting to examine the localization of *axin2* within the cell, as previous work has demonstrated the importance of localization
for activation of particular proteins of the Wnt pathway, in particular nkd1 (Van Raay et al., 2011). Additionally, co-localization studies with other partners within the Wnt signaling cascade along with interaction studies would improve our understanding of Wnt signaling dynamics and how it truly functions to regulate itself. A better understanding of how Wnt signaling is regulated has important implications in development, as we have demonstrated, but can also be used to help understand instances of dysregulation and disease. Dysregulation in Wnt signaling appear to initiate tumor growth within colorectal cancer (Yan et al., 2001; Reya and Clevers 2005; O’Brien et al., 2007). Specifically, it is canonical Wnt signaling that has been identified as being dysregulated within stem cell-like cells which result in colon tumor formation (Baker et al., 2000; Reya and Clevers 2005). With a better understanding of how Wnt signaling is regulated, it is my hope that my findings will help elucidate how instances of Wnt signaling dysregulation arise and provide potential mechanisms for re-establishing its regulation.
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