THE MANY ROLES OF 2-O-SULFOTRANSFERASE IN EARLY ZEBRAFISH DEVELOPMENT

by

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ABSTRACT

Heparan sulfate proteoglycans (HSPG) are a family of cell surface and extracellular matrix proteins with glycosaminoglycan (GAG) chains covalently attached to a protein core. O-sulfotransferases modify these GAG chains by catalyzing the transfer of a sulfate to a specific position on HSPG GAG chains. Although the role of specific HSPG modifications have been described in cell culture and invertebrates, little is known about their functions or abilities to modulate specific cell signaling pathways in vertebrate development. My thesis research focused on the many roles a particular GAG chain modifying enzyme, 2-O-sulfotransferase (2-OST), plays in early development.

To characterize the function of 2-OST in early zebrafish development we knocked down this gene via morpholino and found that 2-O-sulfation controls epiboly. Epiboly is the process by which the cells of the blastodisc move towards the vegetal pole from the animal pole to envelop the yolk cell. Knocking down 2-OST results in a failure to initiate and progress through epiboly due to alterations in assembly of filamentous actin, in microtubule organization, and in yolk cell endocytosis. Syndecans 2 and 4, HSPG core proteins, appear to be targets of the 2-OST activity which contribute to this phenotype.

2-OST activity modulates the activity of multiple signaling cascades. We found that 2-OST is an essential component of canonical Wnt signaling in zebrafish development. 2-OST deficient embryos have decreased β-catenin and E-cadherin protein levels, reduced cell adhesion, and altered cell cycle regulation. The cell cycle and

adhesion defects in 2-OST-deficient embryos can be rescued by reactivation of intracellular Wnt pathway components, but not by overexpression of Wnt8 ligand. Together these results indicate that 2-OST functions within the Wnt pathway downstream of ligand signaling and upstream of intracellular localization and function. Knocking down 2-OST also affects FGF signaling. While Wnt epistasis experiments show loss of 2-O-sulfation impairs some signaling interactions at the cell surface, epistasis experiments with components of the FGF signaling cascade suggest loss of 2-O-sulfation in some contexts can promote signaling which occurs at lower levels in regular developmental contexts. These results suggest HSPG function is essential for early development.

For my parents

who have always encouraged me to go confidently in the direction of my dreams

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CHAPTER 1

INTRODUCTION

The process by which an embryo begins as a single cell and develops into a fully formed organism is regulated by an intricate series of events that can be distilled down to one word: communication. The ability of a single cell embryo to send and receive communication from its environment allows that single cell to divide and become multiple cells. Communication via signaling pathways among multiple cells allows these cells to differentiate and organize the body axes and germ layers. Finally, communication cues from the germ layers and body axes permit the formation of a complex organism containing specialized tissues and organs.

Examination of a wide range of embryos from different species at similar stages in development reveals similar body plans. The conserved pattern of development across vertebrates suggests that the effort to elucidate the signaling pathways that control different processes in development in one organism may yield useful information about the development of other species. Well before the embryo forms a spinal cord or heart, countless signaling cascades are initiated to get from a single cell to the point when the embryo begins to organize itself into a basic body plan. Without these countless interactions the embryo will not organize correctly and will not be viable for long. This dissertation will focus on the events that occur in the first few hours of zebrafish

development and will hopefully foster a greater appreciation of the importance of the earliest forms of communication between cells in the developing embryo.

Stages of Early Zebrafish Development

Cleavage Stage

Zebrafish embryos are externally fertilized. Prior to fertilization, the initial asymmetric animal-vegetal orientation of the embryo is established (Abrams and Mullins, 2009). Maternally deposited proteins and RNA are mobilized in this process with the cell mass forming at the animal pole and vegetal pole forming at the opposite end of the cell mass. Fertilization results in a calcium wave propagated via inositol 1,4,5-triphosphate (IP3) signaling which promotes the segregation of the cytoplasm from the yolk to form a single cell blastodisc at the animal pole (Mei et al., 2009). From this single cell the whole of the developing zebrafish embryo will arise while the yolk contributes nutrients, structural support, and signaling events to guide the orientation of the developing fish. The single cell blastodisc atop the yolk cell begins to undergo many rounds of division, referred to as discoidal meroblastic cleavage because while the genetic material undergoes mitosis and the cells subsequently undergo cytokinesis, the yolk cell does not (Kimmel et al., 1995). Small junctions exist between the cells of the blastodisc, the blastomeres, as well as between the yolk and the cells during the first several divisions, allowing the movement of small molecules between them (Kimmel and Law, 1985c).

Blastula Stage

The first twelve rounds of cleavage occur synchronously in both a meridonial and equatorial fashion atop the yolk, with rapid divisions and small gains in the overall volume of the developing blastodisc. Significant changes begin to occur at the 256 cell

stage, during the 8th and 9th cleavage. Cells in the blastodisc at the margin of the yolk fuse with the yolk and extrude their cellular contents into the yolk (Kimmel and Law, 1985a). This creates the yolk syncytial layer (YSL), a multinucleated cytosolic ring that is not divided by a membrane. The small junctions that had existed between the yolk and the blastodisc are now closed (Kimmel and Law, 1985b). The nuclei within this syncytium, referred to as the yolk syncytial nuclei (YSN), are mitotically active and undergo several more rounds of division within the yolk. Although they are no longer mitotically active they do remain transcriptionally active.

At about the same time as the YSL is forming, movement initiates in the blastodisc as well. This is the first separation of layers since the initial blastodisc establishment began, and starts with the formation of the enveloping layer (EVL). Like a cap on top of the yolk, this most superficial layer of cells in the blastodisc does not give rise to the embryo proper. The cells beneath the EVL are referred to as the deep cells and they will give rise to the germ layers of the embryo proper. By approximately the tenth division, the rate of cleavage begins to slow as a shift occurs in the translation of RNA. Prior to this point, most protein is translated from maternally deposited RNA, but at about 2.5 hours post fertilization (hpf), zygotic gene transcription begins defining the midblastula transition (MBT) (Kane and Kimmel, 1993).

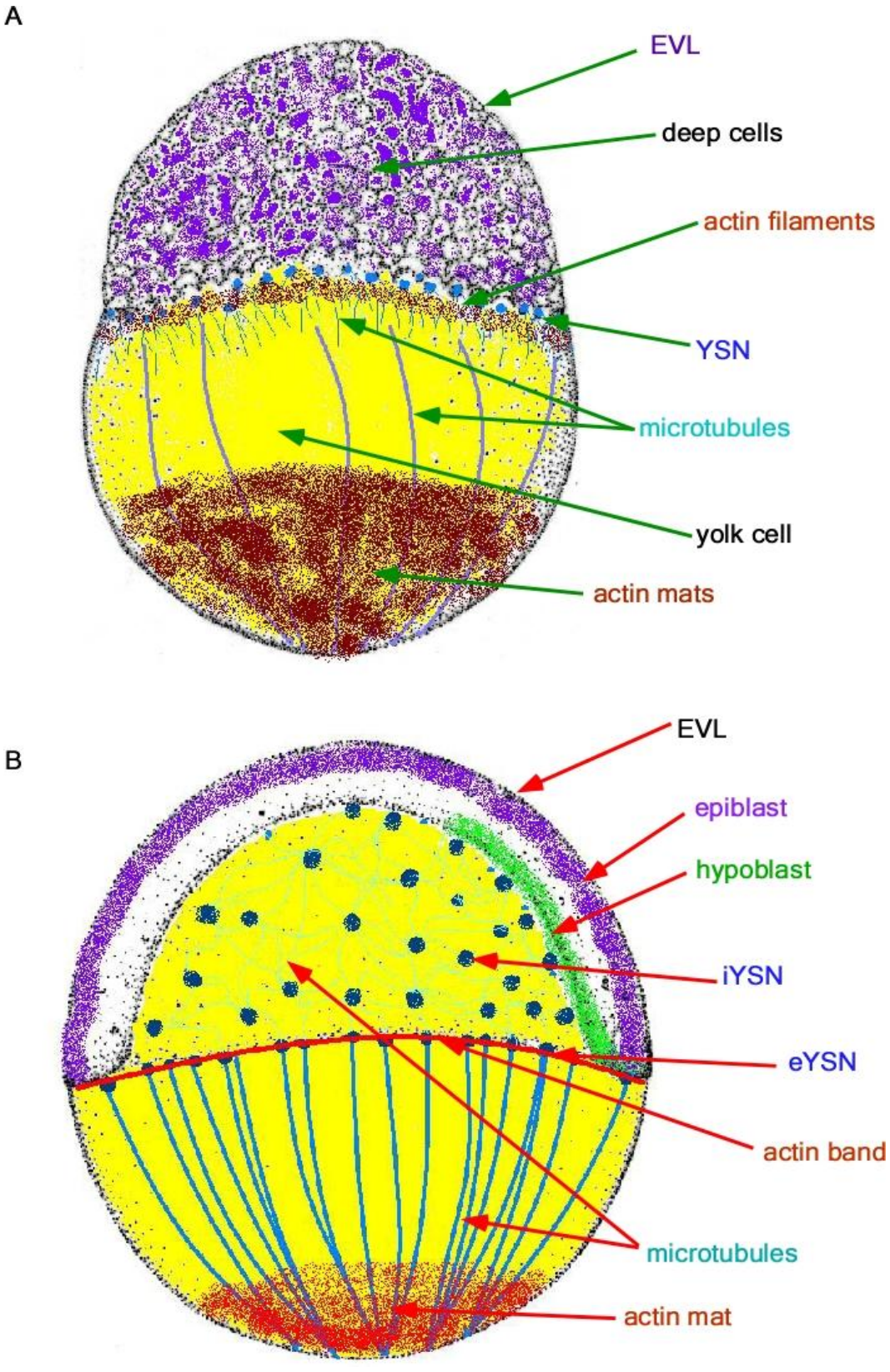
Gastrula Stage

Following the major changes in the organization of the blastodisc come the developmental steps that begin the organization of the body axes and germ layers to form the embryo. Using the three layers established in the blastula stage (the EVL, deep cells, and YSL), the set of processes cumulatively known as gastrulation begin. This stage is

arguably the most important in development (Wolpert, 1978). The first step in gastrulation is known as epiboly, during which the cells atop the yolk migrate towards the vegetal pole to envelop the yolk. The EVL is attached to the YSL and as the EVL margin progresses towards the vegetal pole, the deep cells fill in between the yolk and the EVL (Fig. 1.1A). Furthermore, the deep cells begin a process known as radial intercalation during which the deep cells go from five to six cell layers thick to two to three cell layers thick.

At 50% epiboly, the EVL and deep cells reach the equator of the yolk (Fig. 1.1B). These cells begin to undergo compaction on the dorsal side, setting up the first obvious break in dorsal-ventral asymmetry. This dorsal compaction takes place as the next steps in gastrulation begin to occur (Schneider et al., 1996). Many of the deep cells also begin to migrate towards the dorsal side of the embryo in a process known as convergence. Cells at the margin are remodeled from a single progenitor germ population, the deep cells, into two progenitor populations, the hypoblast and the epiblast. The cells begin to ingress at the margin, migrating first down along the underside of the EVL and then subsequently migrating towards the animal pole along the yolk in a process known as extension. The cells that do not ingress are the epiblast, which will give rise to the ectoderm. The ingressing cells are the hypoblast and are fated to become the mesoderm and endoderm. As the cells of the hypoblast continue to converge, involute, and extend, cells towards the dorsal side tend to establish the more anterior structures. The non-converging epiblast cells from the ventral side tend to contribute to the more posterior

Figure 1.1. Pertinent cellular components during gastrulation. Cell layers and cytoskeletal components of embryo at 4 hpf (A). Cross section of cell layers and cytoskeletal components at 50% epiboly (B).


A
EVL
deep cells
actin filaments
YSN
microtubules
yolk cell
actin mats
B
EVL
epiblast
hypoblast
iYSN
eYSN
actin band
microtubules
actin mat

structures at the vegetal pole. Gastrulation formally ends when the EVL margin reaches the vegetal pole, closing the blastopore and completely enveloping the yolk.

Players in Epiboly

The process of epiboly was first described by the embryologist Karl Ernst von Baer in 1835 (von Baer, 1835). However, it was not until another scientist, John Trinkaus, attempted to tackle the question of how epiboly worked, using the killifish *Fundulus*, that this process became an area of broader interest to the scientific community (Betchaku and Trinkaus, 1978). Trinkaus was interested in the mechanisms which controlled the movement of cells to form organs, and the study of gastrulation provides a readily observable opportunity to understand how largely homogenous cells can migrate and become fated to contribute to everything from heart to brain to skin in just a few hours' time. Only a handful of studies have focused thus far on the initiation and progression of epiboly. This introduction will focus on what is known thus far about the cellular constituents and genes involved in the complex cellular dialogue controlling this process and what questions remain.

Yolk Syncytial Layer and Other Yolk Components

The YSL is an extra-embryonic tissue thought to contribute to several different aspects of early development through a variety of mechanisms including cytoskeletal remodeling, endocytosis, and ligand signaling (Betchaku and Trinkaus, 1978; Kimmel and Law, 1985a). The YSL contains several different cytoskeletal constituents including two arrays of microtubules. The first microtubule array connects the YSN in a cortical array under the margin at the beginning of epiboly and extends underneath the blastodisc as epiboly progresses. The second array radiates from the cortical ring at the margin

down to the vegetal pole. The YSN, which were extruded into the yolk prior to this point, have undergone several rounds of replication and make up two different populations of nuclei as epiboly progresses, though both remain connected through development to the large network of microtubules.

The YSN that migrate underneath the blastodisc are suggested to contribute to the force of the doming process. Studies have demonstrated that these internal YSN (iYSN), the nuclei which migrate underneath the blastodisc, engage in movements similar to the deep cells undergoing convergence and extension overhead (Kimmel and Law, 1985c). This suggests that either the YSN were directing the movements of the deep cells in the blastodisc, or else the cells of the blastodisc are somehow attached to the YSN and dragging the YSN along with them. Elegant studies by the Heisenberg lab suggest that it is the latter case: the YSN and microtubules are drifting in the yolk through cortical flow regulated by the deep cells and attached via junctions to the converging and extending deep cells of the blastodisc (Carvalho et al., 2009).

Microtubules that extend from the YSL to the vegetal pole are believed to actively contribute to epiboly by drawing the EVL towards the vegetal pole as the microtubules shorten (Solnica-Krezel and Driever, 1994). This suggests that the two different groups of YSN, the iYSN and the eYSN, might be controlled by different forces during gastrulation. Indeed, later studies have shown that the iYSN movements depend upon mesendoderm precursors in the blastodisc (Carvalho et al., 2009).

The importance and autonomy of the microtubules have been examined in a variety of ways. Treatment with cold temperatures, nocodazole, or exposure to ultraviolet irradiation all destabilize the microtubules, resulting in stalled epiboly of the

YSL, EVL, and deep cells (Strahle and Jesuthasan, 1993; Solnica-Krezel and Driever, 1994). Treatment with taxol to hyperstabilize the microtubules resulted in a similar block to the epibolic movements of all three layers. Surgically separating the blastodisc from the yolk does not prevent the progression of YSL epiboly, suggesting that the blastodisc is not necessary for the stability of the microtubules (Betchaku and Trinkaus, 1978).

Another cytoskeletal element present in the yolk and crucial to the success of epiboly is filamentous actin. Early in gastrulation actin is found in two populations, in loose filaments at the margin and in a large mat at the vegetal pole (Zalik et al., 1999). When the advancing EVL margin reaches the equator, the actin organization is altered, forming three populations near the margin which are thought to help progression through epiboly by contracting like a drawstring. This subsequently helps constrict the EVL edge as it passes the equator of the yolk and moves towards the vegetal pole (Cheng et al., 2004). A massive amount of remodeling in the cells of the EVL is necessary as they go from the maximum amount of surface area at the equator of the yolk to closure at the vegetal pole; it is these various populations of actin that are thought to help play a role in facilitating that process. Two groups are found in ring-like organization at the interface between the yolk and the blastodisc. One ring is found at the interface of the YSL and EVL at the margin, and the other ring is found between the YSL and the most superficial marginal deep cells. The third population is found in the YSL more vegetal to the cellular interface (Cheng et al., 2004).

All three populations of actin are necessary for epiboly. Experiments with cytochalasin B, an actin destabilizing compound, have shown that all epibolic movements are slowed and the actin populations lost upon treatment, resulting in a failure to

complete epiboly and subsequent death by yolk lysis (Cheng et al., 2004). Treatment with the calcium chelator dibromo-BAPTA completely arrests epiboly and also results in the disruption of these actin populations, resulting in death by lysis as well (Cheng et al., 2004). The drawstring mechanism suggests myosin might be involved in the constricting function of these actin bands. Koeppen et al. used blebbistatin to suppress myosin II function midway through epiboly and found that doing so interrupted the vegetal movements of the EVL, deep cells, and YSL, either by slowing it down or leading to its complete arrest (Koppen et al., 2006). This suggests that the actin rings are mediating their function in epiboly by myosin II.

Another process occurring in the yolk mediated by the cytoskeleton is the internalization of small bits of the membrane from the surface of the YSL which are then transported to the animal pole of the yolk (Betchaku and Trinkaus, 1986). The endocytosis of membrane is thought to reduce the amount of surface area over which the EVL must travel, while also rearranging the overall distribution of yolk in the yolk cell. Disrupting microtubule stabilization interrupts this endocytosis, suggesting either endocytic vesicle formation or the ability to transfer it within the yolk is impaired. The main function of this process in epiboly remains to be confirmed.

The Enveloping Layer

The Enveloping Layer (EVL) is a superficial epithlium that does not directly contribute any cells to the embryo proper. However, cells from the EVL do give rise to the periderm as well as the dorsal forerunner cells (Cooper and D'Amico, 1996; Essner et al., 2005; Oteiza et al., 2008). During the progression through epiboly, the cells of the EVL flatten and elongate, dramatically increasing their surface area (Zalik et al., 1999).

They also move away from the margin while remaining in the EVL layer (Keller and Trinkaus, 1987).

The EVL is generally thought to be towed through epiboly by tight junctions adhering to the YSL, but it is still unclear if the expanding and flattening cells of the EVL play a more active role in driving epiboly (Koppen et al., 2006; Siddiqui et al., 2010). Studies where proper microtubule function is interrupted show that epibolic movements of all the layers in the blastodisc have arrested; however, loss of microtubule function does not completely prevent all expansion and movement of the EVL and deep cells towards the vegetal pole. Thus, many think the EVL and deep cells may play an active role in this process.

The Deep Cells

The deep cells move during epiboly in ways that appear both passive as well as dynamic. After the initiation of epiboly, the deep cells undergo radial intercalation during which they thin from several to just two or three cell layers thick. Deeper cells intercalate into the outer layers which leads to the expansion of the surface area of the deep cells (Kane et al., 2005). This may help drive the doming process. Deeper cells also fill in the space between the EVL and the yolk as the EVL is drawn down towards the vegetal pole. However, it remains unclear whether expansion of deep cells helps actively drive the doming of the yolk or whether the deep cells intercalate more passively due to the force of the yolk doming.

The process of radial intercalation is believed to be mediated by differential adhesion. Studies by Townes and Holtfreter in the 1950s demonstrate that cells expressing a variety of surface markers arrange themselves in the most energetically

favorable clusters, with cells most frequently sorting and subsequently adhering to other cells expressing the same type of markers (Townes and Holtfreter, 1955). It is therefore possible that gradients of adhesive molecules on the surface of the cells may be contributing to these movements (Kane et al., 2005).

The deep cells, which turn into precursor germ layers, have also been shown to be influenced by signals from the yolk cell. Studies have shown that Nodal/TGFβ signals from the yolk are necessary to induce mesoderm and endoderm in the blastodisc at later stages of gastrulation (Rodaway et al., 1999). In another study, RNAse was injected into the yolk to degrade all transcripts (Chen and Kimelman, 2000). This loss of transcription had two effects: it arrested the embryos in epiboly, and it altered the fates of the mesoderm in the blastodisc. Therefore, signals from the yolk are essential for epiboly as well as for proper cell fate in the blastodisc. However, not all mesendoderm induction is dependent on the yolk, as this study also demonstrated that yolk-independent signaling cascades contributed to mesendoderm induction. Embryos also fail to initiate epiboly when treated with α-amanitin which blocks initiation of transcription, restricting protein expression to maternally deposited RNA (Kane et al., 1996). Furthermore, other studies using a mutant line deficient in mesendoderm progenitor cells demonstrated that mesendoderm convergence is essential for iYSN convergence and in the absence of this population of germ cells YSN mediolateral convergence is impaired (Carvalho et al., 2009). This suggests that the blastodisc is imperative for iYSN movements but is unnecessary for eYSN epiboly. It has also been suggested that chemoattractants from the deep cells help modulate iYSN movements. However, no specific evidence has been

offered to support this notion (Cooper and Virta, 2007). Therefore, it appears that dialogue between the yolk and deep cells is crucial for proper development.

Calcium

Calcium signaling is vital to the development of most organisms (Jaffe, 1999). As a small charged molecule, it can easily pass through ion channels and gap junctions, permitting signal modulation over a much greater distance and more rapidly than the rate of diffusion for most other signaling molecules. Calcium may also modulate its effects through IP3 signaling (Slusarski and Pelegri, 2007). In early zebrafish development during the cleavage stage, small intracellular calcium waves have been observed (Webb and Miller, 2006). As development progresses, these waves become larger, covering more area. Studies have demonstrated that calcium signaling is necessary for the initiation of epiboly (Cheng et al., 2004; Sharma et al., 2005). By the beginning of gastrula stage, calcium waves originate from the dorsal side of the embryo and travel across the blastodisc, presumably by gap junctions. At the same time, smaller more focused waves are occurring near the migrating margin. It is thought that the dorsal sided waves may serve as a chemotactic indicator to promote converging cells to move laterally towards the dorsal wide (Webb and Miller, 2006).

Interfering with calcium signaling has devastating effects upon development. Treatment during gastrulation with the chelating agent EGTA results in lysis of the yolk and dissociation of the cells of the blastodisc (Zalik et al., 1999), which impairs epiboly progression. Injecting 5-5'-dibromo-BAPTA into the dorsal side of the yolk at the shield stage blocks calcium signaling in the YSL and results in epiboly arrest because the actin organization is interrupted and fails to form the correct ring structures needed to contract

the marginal EVL (Cheng et al., 2004). Other genes which modulate calcium signaling are likely to be shown to play a role in modulating epiboly.

Overall, these mechanical and chemical disruptions reveal several interesting things about the initial cell layers in zebrafish development. They allow identification of some of the important interactions regulating epiboly. They demonstrate that the activity of these three layers can be uncoupled. Lastly, they provide an opportunity to target specific genes which are known to interact in cell adhesion, migration, and cytoskeletal organization to see if they contribute to epiboly as well as a framework for how to characterize genes that unexpectedly display defects in epiboly.

The Modulators of Communication in Epiboly

Various mutants defective in epiboly have been identified, although the sources of the individual defects have yet to be uncovered for all of them. In spite of several random insertional or base pair mutagenesis screens, fewer epibolic mutants have been identified than expected, given all the different proteins generally thought to interact with and modulate the cytoskeletal elements that regulate epiboly. An example of this is the screen performed by Kane et al. for epiboly mutants (Kane et al., 1996). Although five mutants with epiboly defects were identified from this screen, upon mapping, all were discovered to be alleles of the same gene. This suggests that there may be compensation or redundancy in many of these pathways, which given the partial duplication of the zebrafish genome, is not unexpected.

An alternative to mutant screens is gene manipulation using morpholinos (MOs), synthetic anti-sense oligonucleotides which bind RNA to prevent translation or splicing machinery. MOs that prevent splicing of RNA can suppress only zygotic transcripts,

because maternally deposited RNA is already spliced. MOs which prevent translation can be used to block both maternally deposited as well as zygotically transcripts. However, this does not completely eliminate all gene products because any maternally deposited protein may linger in the developing embryo for an unknown length of time, making it difficult to ever completely eradicate gene function at the stage in development upon which these studies focus. Despite those limitations, both mutants and morpholinos have been helpful in identifying genes involved in epiboly progression and initiation.

Intercellular Components Contributing to Epiboly

Extracellular Matrix

Components of the extracellular matrix (ECM) are thought to contribute to the majority of developmental cell movements. Polysaccharides and proteins fill the space between cells, contributing structural support, a matrix for adhesion or traction necessary for cell migration, and serving as a reservoir for growth factors. The components of the extracellular matrix establish gradients to promote signaling or may be modified or degraded by other components of the matrix. All these different activities promote communication between the cells. The ECM plays major roles in processes similar to epiboly, including dorsal closure in flies, wound healing in all species (Harden et al., 2002; Jacinto et al., 2002; Diegelmann and Evans, 2004), and there may be conserved similarities in the interactions between the cells and the ECM.

Fibronectin (FN) is a protein in the ECM which interacts with integrins and collagen to remodel the cytoskeleton to alter polarity, adhesion, and migration. Studies in frogs and carp have demonstrated a role for FN in epiboly when using antibodies to interfere with FN's ability to bind other components (Gevers et al., 1993; Marsden and

DeSimone, 2001). Two FN genes have been identified in zebrafish. Although FN protein is maternally deposited, FN fibrils are not detectable by immunohistochemistry (IHC) until about 65% epiboly (Trinh and Stainier, 2004; Latimer and Jessen). A maternal-zygotic mutant for one *FN* gene, and a partial knockdown by morpholino against both gene copies, result in defects in convergent extension during gastrulation but fail to demonstrate an earlier phenotype. However, as neither is a complete knockdown based on IHC, there is still a reasonable possibility that FN could be contributing to epibolic progression. Furthermore, as normal actin organization begins to be reorganized at the same time that FN begins to undergo larger scale fibrillogenesis, there is still a possibility that FN contributes to epibolic movements.

Cell Membrane

Membrane microdomains are lipid rafts found in the fluid bilayer of all types of cells. These regions serve as "signaling hotspots" by localizing several different adhesion and signal cascade initiating elements (Simons and Ikonen, 1997). Studies in Medaka suggest that membrane microdomains may play a powerful role in epiboly (Adachi et al., 2007). Disrupting the ability of these domains to interact between cells in the developing embryo using methyl-beta-cyclodextrin (MBCD) impairs epiboly in Medaka by disrupting the localization of certain adhesion molecules and demonstrates a requirement for calcium signaling in this process (Adachi et al., 2009).

Many steps in development are conserved between Medaka and zebrafish. However, it is unclear if the role of membrane microdomains in epiboly is one of those. Many of the elements generally conserved across organisms in these detergent-insoluble lipid rafts have been demonstrated to play a role in epiboly regulation. Signaling

elements localizing to membrane microdomains include E-cadherin, beta-catenin, actin, phospholipase C gamma, Src, and various glycoproteins and glycolipids (Maxfield, 2002). These signaling hotspots promote localization of many epiboly regulating proteins, though it is unclear whether these proteins localize to membrane microdomains. Therefore, it is possible that these regions help organize and promote some of this communication which regulates epiboly.

Adhesion

Adhesion plays a crucial role in epiboly as well as development at large. Adhesive forces that are too strong will prevent the necessary movements needed to organize the developing organism, and weak adhesion prevents cells from communication with each other as well as engaging in the correct migration patterns (Spargo et al., 1994). Several different adhesion molecules have been shown to play a role in regulating epibolic movements so far. Tight junction associated proteins, such as Zo-1 and claudins, connect EVL cells to each other and the EVL to the YSL (Koppen et al., 2006; Siddiqui et al., 2010). Adherens junctions are found between the EVL and deep cells as well as between the deep cells themselves (Babb and Marrs, 2004).

Tight junctions have long been hypothesized to play a role in epibolic regulation by connecting the EVL to the YSL. However, little has been done to confirm this idea (Koppen et al., 2006). The first study to address this through genetic rather than physical manipulation looked at ClaudinE (Siddiqui et al., 2010). Epiboly was impaired in a dosage dependent fashion, with lower doses resulting in epibolic delay and higher doses resulting in a complete failure to initiate epiboly. This is surprising because 23 claudin genes have been annotated thus far in the zebrafish genome and it is not unreasonable to

expect that the absence of one claudin might lead to compensation by others (Siddiqui et al., 2010). Furthermore, claudins have previously been demonstrated to require zonula occludins proteins (Zo1 and Zo2) for their localization to tight junctions (Koval et al., 2010). However, the Zo proteins do not seem to require claudinE for their proper localization. Zo proteins have been shown to localize to membrane microdomains (Nishiyama et al., 2001), suggesting that tight junctions and redundant claudin localization may be important to this epibolic process.

The most well characterized adhesion molecule that contributes to epiboly is E-cadherin, a component of adherens junctions. This transmembrane homotypic-adhesion molecule's role in epiboly was first identified in a screen for epiboly mutants (Kane et al., 1996). Since this study, several other upstream mediators of E-cadherin levels and localization have been identified. Mutants of *E-cadherin* undergo delayed epiboly in the EVL but eventually complete the process (Babb and Marrs, 2004; Kane et al., 2005). The defect lies in the deep cells, which initially undergo epiboly with the EVL, even undergoing radial intercalation (Shimizu et al., 2005). However, shortly after these cells thin out to spread the deep cells, they de-intercalate, lose their morphology and fail to continue epiboly.

The E-cadherin epiboly defect is significant for several reasons. It was one of the earliest examples of the fact that the EVL and deep cell epibolic movements can be uncoupled, suggesting they are not completely dependent upon one another. It also demonstrates that radial intercalation, which had not previously been recognized as an important force, is crucial for epiboly. Lastly, it provides support for the idea of differential adhesion because E-cadherin levels are, in WT embryos, expressed in a

gradient from 30% epiboly on, with greater levels of E-cadherin localized to the membrane in the EVL and more superficial deep cells compared with the innermost layers. Therefore, the de-intercalation observed in the morphants and mutants may be due to an alteration in gradients, which reduces the energetic motivation to sort into fewer layers.

Another single-transmembrane cadherin has been demonstrated to contribute to epiboly as well (Aamar and Dawid, 2008). Both overexpression and morpholino knockdown of protocadherin 18a result in impaired later-stage epiboly. Too much protein causes too much adhesion, while too little prevents the cells from adhering well enough to promote adhesion.

Another atypical cadherin, Celsr, a homologue of the *Drosophila* Flamingo, is a seven-pass transmembrane protein which has been implicated in regulating convergence extension movements during gastrulation in fly development. Three *celsr* genes have been identified in fish which are ubiquitously expressed early on and functionally redundant (Formstone and Mason, 2005). Morpholino knockdown of two genes, celsr1a and celsr1b, in a maternal-zygotic *celsr2* null mutant background results in a late epibolic arrest in which the blastopore fails to close (Carreira-Barbosa et al., 2009). While not lethal at this stage, unlike many epiboly defects, this phenotype is significant because noncanonical Wnt genes had not been previously implicated in epibolic regulation. To further explore how *celsr* genes may be contributing to epiboly, the authors devised constructs which separate the protein function of the intercellular and extracellular regions. They discovered that celsr modulates epiboly in a noncanonical Wnt-

independent fashion through its extracellular domain mediating cohesion between cells. The cytoplasmic domain had no effect on epiboly.

Proteins which interact in some way with E-cadherin are another likely source of epiboly defects. Epithelial Cell Adhesion Molecule (EpCAM), a single-pass transmembrane glycoprotein, is a gene most frequently studied in conjuction with metastasis and other aspects of cancer progression. Elucidating its function has been difficult because in some contexts it appears to support adhesion, while in others it appears to interfere with it (Winter et al., 2003; Baeuerle and Gires, 2007). During gastrulation in the zebrafish its expression is restricted to the EVL. *EpCAM* maternal-zygotic mutant embryos display defects in late epiboly via slowed marginal migration (Slanchev et al., 2009). Authors of this study propose this delayed progression is due to reduced localization of E-cadherin to the EVL cell membrane, reduced basal lamellopodial protrusions, but expanded expression of tight junction components basally. If E-cadherin is knocked down *via* morpholino in the *Epcam* MZ background, then the EVL and deep cells completely detach. This effect suggests that EpCAM and E-cadherin interact in a functionally redundant fashion in the EVL to support the adhesive integrity of that cell layer.

Some proteins localized to the cell surface have produced epiboly defects through unexpected mechanisms. The proteins, which when misfolded cause neuroencephalopathies in mammals, such as scrapie, mad cow disease, and Creutzfeld-Jakob Disease (Prusiner, 1991), are often expressed in many tissues. This suggests they may have an important function. To date, however, studies to determine their normal function have not led to definitive answers. The mouse Prion Protein (Prp) is broadly

expressed, yet the knockout is viable without any obvious defects aside from the inability to contract scrapie (Bueler et al., 1992). Two prion related genes have been found in zebrafish, one of which is expressed ubiquitously during gastrulation. Morpholino knockdown of this membrane-tethered glycoprotein, Prion protein related sequence 1 (Prnprs1), results in an epiboly defect (Malaga-Trillo et al., 2009). GFP-tagged Prnprs1 appears to localize to the cell membranes in a pattern suggesting it mediates homotypic cell adhesion. In these morphants, E-cadherin failed to localize to the cell membrane correctly most likely causing the radial intercalation defect observed in the deep cells of the blastodisc which presumably contributes to the epiboly defect. The reduced E-cadherin levels may possibly contribute to the increased cytosolic levels of B-catenin and they speculate that reduced Src activity may be contributing to this phenotype as well. *Prp* genes have been demonstrated to localize to membrane microdomains and thus potentially some element of this interrupted signaling is due to that localization (Solis et al., 2010).

Another interesting study with an unexpected outcome looked at the calcium channel β subunit proteins belonging to the Membrane Associated GUanylate Kinase (MAGUK) family, CACNβ4.1 and β4.2 (Ebert et al., 2008), which associate with α subunits to form the transmembrane channels. Morpholino knockdown led to several classifications of epiboly defects, with the most severe failing to even initiate epiboly. As calcium has already been demonstrated to play an important role in epiboly, the fact that altering one of the voltage sensitive ion channel components resulted in epiboly defects is not surprising. What is surprising is that when different rescue constructs based on different parts of the genes are coinjected with the morpholino, it is the construct lacking

the region which associates with the α subunit, thus failing to rescue the calcium channel, which rescues the epiboly defects. This suggests these genes have some previously unrecognized function besides their role in calcium channel formation, perhaps by stabilizing the microtubules or tight junctions.

One of the advantages to using zebrafish as a model is that it allows exploration of developmental roles for genes which have been previously well established in other model systems, but are found to have a different, context dependent role in development. In mice, knockout of the *apelin receptor* results in aberrant fluid homeostasis (Roberts et al., 2009). Two copies of the *apelin receptor* have been identified in fish, *aplnra* and *aplnrb*. The latter has been shown to play a role in myocardial progenitor formation and migration (Scott et al., 2007; Zeng et al., 2007). Morpholino knockdown of aplnra, the expression of which begins prior to the initiation of epiboly, results in a failure to complete epiboly, with the stage of development at which the migration of the EVL and deep cells arrest occurring in a dose-dependent fashion (Nornes et al., 2009). However, it does not seem to arrest the progression of the YSN, suggesting a detachment between adhesion of the YSN and the EVL in the aplnra MO embryos. As apelin, the ligand for this receptor, is not expressed until several hours after the initiation of the receptor, and as ectopic expression of aplnra also results in epiboly defects, the implication is that misregulated expression of aplnra is altering epiboly through some means other than the previously established ones.

Intracellular Components Contributing to Epiboly

Scaffolding Proteins and Their Partners

Not only do elements spanning the cell membrane contribute to epiboly, but so do many different types of proteins inside the cell. Scaffolding proteins, which associate with some of the transmembrane proteins which localize to the membrane microdomains, also contribute to epiboly. Angiomotin-like 2 (Amotl2), belongs to a family of proteins which regulate endothelial cell motility (Huang et al., 2007). Knocking down Amotl2 in fish results in a dose-dependent delay in epiboly and epiboly arrest at high doses. This results in less abundant and more disordered actin in the yolk, more rounded EVL cell morphology suggesting reduced tension between the yolk and EVL, and reduced membrane protrusions. This gene was identified in an FGF responsive microarray screen (Huang et al., 2007) and co-localized in cell culture with phosphorylated Src, though further studies need to be performed to determine if this result has any bearing in vivo.

Another family of cytoskeletal proteins that interact with scaffolding proteins and seem to play a role in epibolic progression are the keratins (Pei et al., 2007). Their role in epiboly was discovered based on a transcription factor morpholino RNA microarray compared to wild-type RNA and validated by *in situ* hybridization results. Knocking down the transcription factor FoxH1 resulted in reduced levels of several keratins. In an attempt to reproduce the transcription factor knockdown, morpholinos against several of the keratins were injected, producing the same mid-epiboly arrest phenotype. How they modulate epiboly is unknown at this point, but further studies will be interesting due to the dynamic relationship between the cytoskeletal elements of keratins, actin, and microtubules. In many instances the stability of one depends on another so perhaps

reduced keratin levels reduce the stabilization of microtubules in the yolk, or discourage the proper formation of the actin contractile ring.

Diaphanous-related formin 2 (Diaph2) is another scaffolding related protein which belongs to a family known to play a role in actin polymerization and contributes to epiboly (Lai et al., 2008). When it is knocked down, actin filamentation is reduced at the leading edge of the deep cells, which probably contributes to the epiboly defect. However, they demonstrated that simultaneously knocking down profilin1 (zpfn1) produced a more penetrant epiboly defect, suggesting they interact synergistically to contribute to this process. As zpfn1 is expressed only in the EVL at this time, it suggests that Diaph2 and zpfn1 perhaps interact in that context to contribute to epiboly through actin assembly, and that the deep cells epiboly defects are secondary. More importantly, as Diaph2 in yeast-two-hybrid has been shown to interact with the small G-proteins RhoA and Cdc42 in their constitutively active form, these interactions are probably mediating part of this cascade.

Small G Protein Cascade

One of the largest sets of families shown to play a role in epiboly are the small G proteins and the proteins that regulate their activity. The small G proteins, which include Racs, Rhos, and Cdc42, cycle between an "off" GDP-bound state and an "on" GFP-bound state. Modulating the activity of these proteins affects a variety of processes including cytoskeletal reorganization. Chimerin1 (Chn1), Chp, and $G\alpha_{12/13}$, all directly or indirectly contribute to their regulation of epiboly *via* guanine nucleotide exchange factors.

Chimerin1 (chn1) is a Rac-GTPase activating protein which, when knocked down, leaves Rac hyperactive in the GTP-bound state, causing embryos to proceed through epiboly more rapidly than normal and resulting in expanded *bmp4* and *sonic hedgehog* expression (Leskow et al., 2006). Restricting Chn1 knockdown to the yolk results in a similar, but more mild phenotypic defect, suggesting Rac activation in the yolk contributes to epibolic regulation in some way. The details of the regulation have yet to be explored and the authors speculate it is due to actin organization.

Chp is an atypical Rho-GTPase bearing homology to Cdc42. E-cadherin and β-catenin fail to localize correctly to adherens junctions at the cell membrane in both deep cells and the EVL when Chp levels are reduced by morpholino knockdown, resulting in a delayed epiboly phenotype similar to that seen in the E-cadherin mutants (Tay et al., 2010). Despite the adherens junctions disruption, the actin and microtubules appear fairly normal. Chp is known to bind the effector kinase PAK, which interacts with Rac1 and Cdc42 (Manser et al., 1994). The authors suggest that Chp signaling occurs upstream of βPIX/PAK as signaling knockdown of all three components yield similar defects. RhoA has been shown to be downstream of Wnt5/11 signaling, modulating convergence and extension, in a manner involving Diaph too (Zhu et al., 2006). This led the authors to suggest Chp acts downstream of Wnt11/5 and upstream of Rab5-mediated endocytosis as the E-cadherin localizes to these vesicles in lieu of the cell surface.

Another family of G proteins shown to contribute to epibolic regulation via E-cadherin through a Rho-dependent pathway is $G\alpha_{12/13}$ of which there are three members, $G\alpha_{12}$, $G\alpha_{13a}$, and $G\alpha_{13b}$ (Lin et al., 2009). Both overexpression of $G\alpha_{13}$ protein and morpholino knockdown of all three proteins simultaneously results in epiboly defects

which manifest at roughly 70% epiboly. These proteins bind to the cytoplasmic tail of E-cadherin, blocking the site for β-catenin to bind, and by this means inhibit deep cell to deep cell adhesion. Not surprisingly, the deep cell epiboly defect resembles the E-cadherin mutants. However, the EVL is arrested as well in the Gα morphants. In E-cadherin mutants, however, the actin organization is fairly normal and it is altered in these morphants. The authors conclude that this cytoskeletal alteration is due to altered actin assembly mediated by an interaction with the RhoGEF based on co-precipitation experiments in cell culture and similar phenotypes overexpressing $G\alpha_{13}$, the RhoGEF Arhgef11, and a constitutively active RhoA.

Biosynthetic Cascade

Another way G proteins are activated is by metabolite signaling. Interfering with the prostaglandin E2 (PGE(2)) biosynthetic cascade, either by morpholino or by chemical inhibition reveals a role for this cascade in epiboly. PGE(2) formation is promoted by a variety of enzymes and intermediate metabolites from arachidonic acid, including cyclooxygenase 1 (COX1) (Cha et al., 2006) and Prostaglandin E synthase (Ptges) (Grosser et al., 2002). At lower doses, morpholinos against these two enzymes result in slowed or delayed epiboly, while higher doses produce a more dramatic epibolic arrest. Treatment with PGE(2) can rescue both of these morpholino-induced defects. However, unlike some other genes which interfere with epiboly if they are knocked down or over expressed, increased PGE(2) has no effect on epiboly.

Studies of cancer cells in culture have demonstrated that PGE(2) modulates metastasis via the activation of the PI3K/Akt pathway (Buchanan et al., 2003). Active Akt levels, a direct target of PI3K signaling, are reduced in ptges morphant embryos (Cha

et al., 2006). Conservation of this cascade is supported by results which demonstrate that knockdown of PI3K by chemical inhibition results in epiboly as well as C/E defects. Therefore, PGE(2) seems to play a role in regulating epiboly via PI3K/Akt signaling in normal development. However, other studies have suggested that PGE(2) modulates epiboly and other gastrulation movements by controlling levels of cell-cell adhesion (Speirs et al., 2010). This is done by stabilization of Snai1a, which normally represses *E-cadherin* transcription. In the absence of proper levels of PGE(2), Snai1a is not properly stabilized, resulting in increased expression of E-cadherin and thus increased cell adhesion. This effect is modulated via glycogen synthase 3 kinase, not β-catenin nor PI3K.

Another biosynthetic cascade which contributes to epiboly involves the conversion of cholesterol to pregnenolone, the first step in the steroid hormone production cascade. Knocking down the early ubiquitously expressed enzyme which mediates this conversion, cyp11a1, results in epibolic delay due to destabilized microtubules (Hsu et al., 2002; 2006). Treatment with pregnenolone rescues the epiboly delay by stabilizing the yolk microtubules, suggesting that cyp11a1 contributes to epibolic regulation by directly or indirectly stabilizing microtubules instead of modulating some downstream signaling cascade.

Kinases

Kinases are enzymes that transfer a phosphate group to a protein. These proteins are often intermediate members of a signaling cascade and the phosphorylation functions to either promote or repress signal transduction. Quite a few kinases have been implicated in epiboly, though not all of their targets are yet identified. However, the

kinases identified thus far have been shown to regulate a range of aspects of epibolic regulation. Several components of the MAPK families as well as Src kinases are involved, though the redundancy as well as novelty have yet to be elucidated (Roux and Blenis, 2004).

The maternal-zygotic mutant *bettyboop* (*bbp*) undergoes normal epibolic progression until it reaches the equator of the yolk (Holloway et al., 2009). The blastodisc shimmies during the first part of epiboly, but upon reaching the equator the actin at the margin undergoes rapid remodeling, resulting in rapid constriction and subsequent embryonic death. The mutation in *bbp* causes a premature truncation in *MAPKAPK2*, a target of one branch of the MAPK family. This kinase is normally activated by phosphorylation by p38 MAPK, but the early truncation eliminates the site p38 normally phosphorylates. This mutant also displays aberrant calcium spikes which they believe promote the actin constriction defect as well as the shimmying prior to that. Intriguingly, *MAPKAPK2 -/-* mice display no early developmental defects (Lehner et al., 2002).

Another branch of the MAPK family which has been shown to play a role in epiboly is ERK2 (mapk1) (Krens et al., 2008). This kinase is activated by FGF signaling. The epiboly defect is manifested in a MO dose-dependent fashion and appears to modulate the cytoskeleton by reducing actin polymerization and microtubule stabilization. The authors speculate that Paxillin, a focal adhesion adaptor, interacts with MEK, an upstream kinase, to coordinate ERK- focal adhesion kinase (FAK)-Rac activation. Knocking down various members of the FGF signaling alone and in combination with other cascades fails to replicate this phenotype, but given the

substantial number of family members there is probably a fair amount of redundancy and compensation. Using a drug which blocks all FGF signaling, SU5402 (Mohammadi et al., 1997), also results in an epiboly initiation defect through similar modulation of cytoskeletal elements.

Tnika, a MAP4K, plays a role in epiboly by regulating both the actin and myosin organization in the yolk (Koppen et al., 2006). Morpholino knockdown of this gene results in epiboly movements arresting at about 65% epiboly. This appears to be due to altered actin and a failure to correctly undergo cell shape changes in the EVL at the margin. Correct myosin localization appears to be necessary for actin recruitment to the margin to form the contractile band in the yolk, although how Tnika contributes to this process remains to be elucidated. In *Drosophila* dorsal closure, this gene functions upstream of the JNK pathway, the third major branch of MAPK signaling, and functions in a similar fashion to modulate cell shape and cytoskeleton remodeling (Su et al., 1998).

In some contexts, Src kinases are thought to signal upstream of the MAPK cascades. Three Src kinases are expressed ubiquitously through early development, Src, Fyn, and Yes. All three are speculated in some contexts to have redundant functions (Tsai et al., 2005). Using a drug, PP2, to knockdown Src kinase family members reveals a role for Src kinases in epiboly initiation. Two Src kinase family members have been shown to contribute to epibolic progression. At higher doses, reducing Yes kinase expression via MO results in early epiboly arrest, possibly due to its interaction based on co-precipitation data, with FAK. Fyn knockdown by MO does not cause an epiboly defect directly, but using a dominant negative construct results in a failure to initiate epiboly due to impaired calcium signaling (Sharma et al., 2005). Knocking down Fyn

and Yes simultaneously by lower dose MO also does not produce an epiboly defect and neither does knocking down Src by MO in conjunction with the other two (Jopling and den Hertog, 2005). Based upon this evidence it seems Fyn and Yes converge upon RhoA in parallel with Wnt5 and Wnt 11 to regulate convergence and extension. Further studies are needed to determine whether these kinases directly contribute to epibolic regulation or whether other homologs of these genes, such as the uncharacterized second isoform of *fyn*, also contribute to the complexity of this pathway.

Transcription Factors

Forkhead Box H1 (FoxH1), a transcription factor downstream of nodal/TGFβ signaling, was shown to contribute to epiboly when knocked down by morpholino, causing epibolic delay at about shield stage (Pei et al., 2007). To identify genes regulated by Nodal/TGFβ signaling via FoxH1, the authors performed a microarray screen to try to identify likely candidates. They found five *keratin* genes down-regulated at the RNA level, and subsequently knocking down all five genes which are normally expressed in the EVL induces a similar phenotype to the FoxHI knockdown. This may be due in part to the failure of the EVL to differentiate and thus function correctly.

Interferon Regulatory Factor 6 (IRF6) is another gene which seems to contribute to epiboly by playing a role in EVL specification and is also a transcription factor (Sabel et al., 2009). Expression of a dominant negative against this gene impairs epiboly, resulting in arrest of the EVL, deep cells, and YSL. The splice blocking MO knockdown, however, did not produce early defects. Furthermore, injection of the *dnIRF6* restricting its activity to the yolk has no effect on epiboly, suggesting that the dynamics between the EVL and YSL must be necessary for epibolic progression.

Aside from directly influencing genes involved in epiboly, transcription factors may regulate other important epiboly transcription factors. For example, the T box factor Eomesodermin A (Eomesa) has been shown to act upstream of Mix Type Homeobox 2 (Mxtx2), and both of them contribute to epiboly (Bruce et al., 2005). A dominant-negative *Eomesa* construct interferes with radial intercalation, thus disrupting epiboly initiation and doming. Mxtx2 has a more mild effect when knocked down by MO, resulting in slowed epiboly which leads to cell lysis of the yolk when the actin drawstring constricts. What is curious is that the actin filaments appear to be missing in the yolk, but the constriction function is apparent, suggesting the actin filaments in the EVL contribute to the process. The microtubules, however, are only mildly disordered. Restricting the MO knockdown of Mxtx2 to the yolk induces a similar though more mild phenotype.

Another transcription factor with a similar phenotype is Pou Domain, class 5, transcription factor 1 (Pou5f1, also known as Oct4) (Lachnit et al., 2008). This gene has been implicated in stem cell self-renewal in mammals, but does not seem to be functioning by the same means in the zebrafish (Pan et al., 2002). This gene is expressed ubiquitously early but becomes restricted to the epiblast during gastrulation. The MZ mutant (MZ*spg*) exhibits delayed doming followed by delayed EVL migration and severe deep cell epiboly defects. Both the actin filaments and microtubule polymerization are reduced and disordered in the yolk. The targets for this gene contributing to the alterations in the cytoskeleton have yet to be identified. It has been postulated that Pou5f1 and Eomesa might function in parallel pathways as both play a role in endoderm fate specification (Bjornson et al., 2005).

One of the confounding issues with transcription factors is that many different aspects of development may be affected. It can be difficult to separate the primary defect contributing to the epibolic defect versus secondary effects which may affect other aspects of development. The Special AT-rich sequence Binding Protein 2 (Satb2) is a transcription factor which, when its expression is blocked by a splice blocking MO, results in epibolic arrest at about 60% epiboly, producing embryos with an elongated shape (Ahn et al., 2010). Endoderm fate specification apparently is not affected unlike the previous transcription factors discussed which contribute to epiboly, but instead seems to affect the migration ability of axial mesoderm. The primary defect appears to be related to impaired endocytosis and exocytosis functions in the cell, perhaps by affecting polarity of the cells and reducing migration. The YSL in these embryos is not formed properly, which might be the primary defect related to epiboly, though altered actin filaments due to a failure to correctly mobilize and remove things from the cell surface seems to be a likely possibility.

Post-translational Modifiers

Modifications to proteins after they have been translated contribute to the complexity of signaling pathways. Many of these modifications occur in a highly dynamic fashion. Two genes which are known to modulate many of the genes already addressed in this review, including *E-cadherin*, transcription factors, *β-catenin*, *myc*, etc., are *O-GlcNAc transferase (Ogt*), which catalyzes the transfer of an O-GlcNAc to a target protein, and *Oga*, which removes the O-GlcNAc group from a protein (Webster et al., 2009). Increasing Ogt or Oga activity via injected RNA delays late phase epiboly, but not the onset or initial progress of the process indicating that O-GlcNAc modifications

control the activity of one or more proteins involved in epibolic regulation. Intriguingly, both overexpression as well as MO knockdown of Ogt result in smaller embryos, increased apoptosis, and failure to specify endoderm as well as some markers of mesendoderm fate, but only the RNA causes the epiboly defect. The microtubules are thicker and shorter and the actin filaments are reduced in both Ogt and Oga overexpression. The authors speculate that Pou5f1 may be a target contributing to this phenotype because O-GlcNAc inactivates this protein and the phenotypes are similar.

Another family of genes involved in the post-translational modification of proteins adds glycosaminoglycan (GAG) chains to protein cores (Esko and Selleck, 2002; Kramer and Yost, 2003; Hacker et al., 2005), creating molecules known as proteoglycans. The type of proteoglycan is characterized by the sugars which comprise the GAG chains. These GAG chains are then modified by different enzymes which epimerize, transfer sulfates, remove sulfates, and remove acetylases, and are subsequently mobilized to the cell surface or extruded into the ECM. From there, these proteoglycans facilitate communication between cells and their environment by promoting ligand interactions, establishing signaling gradients, and contributing to the structure of the ECM.

The studies that follow focus on a particular type of proteoglycan family, the heparan sulfate proteoglycans (HSPGs). Many questions remain regarding not just which genes control epiboly, but which processes contribute to the vegetal movement of these cells, what actually promotes the initiation of epiboly, what is actively moving in epiboly and what is passively being moved by something else, and what other unidentified genes come into play as well. Due to the localization of HSPGs to the cell surface, we believe

they may play an important role in cellular communication in early development. To address these questions I will examine the way altering the modifications to the GAG chains by knocking down the activity of 2-O-sulfotransferase affects cellular behavior. I will also try to identify which core proteins have GAG chains whose modifications by 2-OST may be contributing to these behaviors. This will help establish which different signaling cascades are modulated by the presence of these modifications which may contribute to the regulation of epiboly. I will build on the base of knowledge that currently exists about how epiboly is controlled to identify the mechanisms controlling epiboly, not just the individual genes which play a role.

Summary

In summary, we know communication between all the layers of the embryo is essential for proper development. Elements located on the surface of the cell as well as within the cytoplasm play important roles in regulating epiboly initiation and progression by controlling behaviors ranging from cytoskeletal organization and function to transcriptional regulation. I will provide insight into the role cellular communication plays in epiboly by examining the effects 2-O-sulfation of GAG chains has upon different signaling cascades and cellular activities in early development.

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CHAPTER 2

2-O-SULFOTRANSFERASE CONTROLS WNT SIGNALING TO REGULATE CELL CYCLE AND ADHESION IN ZEBRAFISH EPIBOLY

Abstract

O-sulfotransferases modify Heparan Sulfate Proteoglycans (HSPGs) by catalyzing the transfer of a sulfate to a specific position on HS glycosaminoglycan chains. Although the role of specific HSPG modifications have been described in cell culture and invertebrates, little is known about their functions or abilities to modulate specific cell signaling pathways in vertebrate development. Here we find that 2-O-sulfotransferase (2-OST) is an essential component of canonical Wnt signaling in zebrafish development. 2-OST deficient embryos fail to initiate epiboly, have decreased β-catenin and E-cadherin protein levels, reduced cell adhesion, altered cell cycle regulation, and fail to respond to exogenous Wnt8. The cell cycle and adhesion defects in 2-OST-deficient embryos can be rescued by reactivation of intracellular Wnt pathway components, but not by overexpression of Wnt8 ligand. Together these results indicate that 2-OST functions within the Wnt pathway downstream of ligand signaling and upstream of GSK3β and β-catenin intracellular localization and function.

Introduction

Heparan sulfate proteoglycans (HSPGs) are a family of cell surface and extracellular matrix proteins with glycosaminoglycan (GAG) chains covalently attached to a protein core. Many types of cell behavior are thought to be modulated by HSPGs, from self-renewal and adhesion to differentiation and migration (Esko and Selleck, 2002; Hacker et al., 2005; Lamanna et al., 2007). The ability of HSPGs to modulate cellular responses is partially controlled by modifications to the GAG chains (Walsh and Stainier, 2001; Reim and Brand, 2006; Gotte et al., 2008; Reijmers et al., 2010).

The GAG chains present in HSPGs are disaccharide repeats, 50-100 units in length on average, composed of alternating glucuronic acid and N-acetyl-glucosamine (Esko and Selleck, 2002). Modifications to these GAG chains by several different families of enzymes epimerize ring structures, add sulfates, and remove acetylases in a nontemplate driven fashion, resulting in variable regions of sulfation on each GAG chain and creating an astonishing level of complexity within one cell-surface HSPG. Gene knockdown and mutants for HSPG components have developmental defects which suggest distinct roles for members of these HSPG modifying enzymes, but none of these studies have identified sulfation-dependent signaling pathways in early development (Bullock et al., 1998; Bink et al., 2003; Kamimura et al., 2004; Habuchi et al., 2007; Fujita et al., 2010).

In this study we focus on one GAG modifying enzyme, 2-O-sulfotransferase (2-OST). 2-OST catalyzes the transfer of a sulfate from a phosphate donor, 3'-phosphoadenosine 5'-phosphosulfate (PAPS), to the second carbon position on the glucuronic acid of the disaccharide chains. Studies in mice and worms previously

demonstrated a role for 2-O-sulfation in cell migration and differentiation through unknown pathways (Bullock et al., 1998; Kinnunen et al., 2005).

The zebrafish begins as a single cell atop a yolk mass, undergoing several rounds of synchronous cell division to form a blastodisc (Kimmel and Law, 1985a; Kimmel et al., 1995). At 4 hours post fertilization (hpf) the process of epiboly begins. At this time the most superficial layer of epithelium, the enveloping layer (EVL), begins a downward migration towards the vegetal pole. The EVL completely surrounds the yolk and epiboly concludes with complete enclosure of the yolk cell (Betchaku and Trinkaus, 1978; Kane and Adams, 2002). The cells of the blastodisc beneath the EVL, known as the deep cells, give rise to the embryo proper through a variety of cell movements during gastrulation (Rohde and Heisenberg, 2007). These embryonic deep cells intercalate and expand vegetally, filling the space between the EVL and the nonembryonic yolk during the epiboly movements (Kane et al., 2005). The extent to which epiboly is driven by the pulling forces of the EVL compared to the expansion forces of the deep cells is still unclear.

Here we examine the role of 2-OST in cell migration, proliferation, and adhesion in zebrafish embryos, and show that the canonical Wnt pathway is dependent on 2-OST. We show that reducing 2-OST levels in zebrafish results in a failure to initiate epiboly. At the cellular level, β-catenin and E-cadherin regulation is altered, resulting in altered cell cycle regulation and altered cell adhesion within the embryonic deep cells. Results from a series of epistasis experiments indicate that cellular effects in 2-OST deficient embryos can be rescued by reactivation the intracellular canonical Wnt pathway,

indicating that 2-OST function is required for normal Wnt signaling in embryonic cells during epiboly.

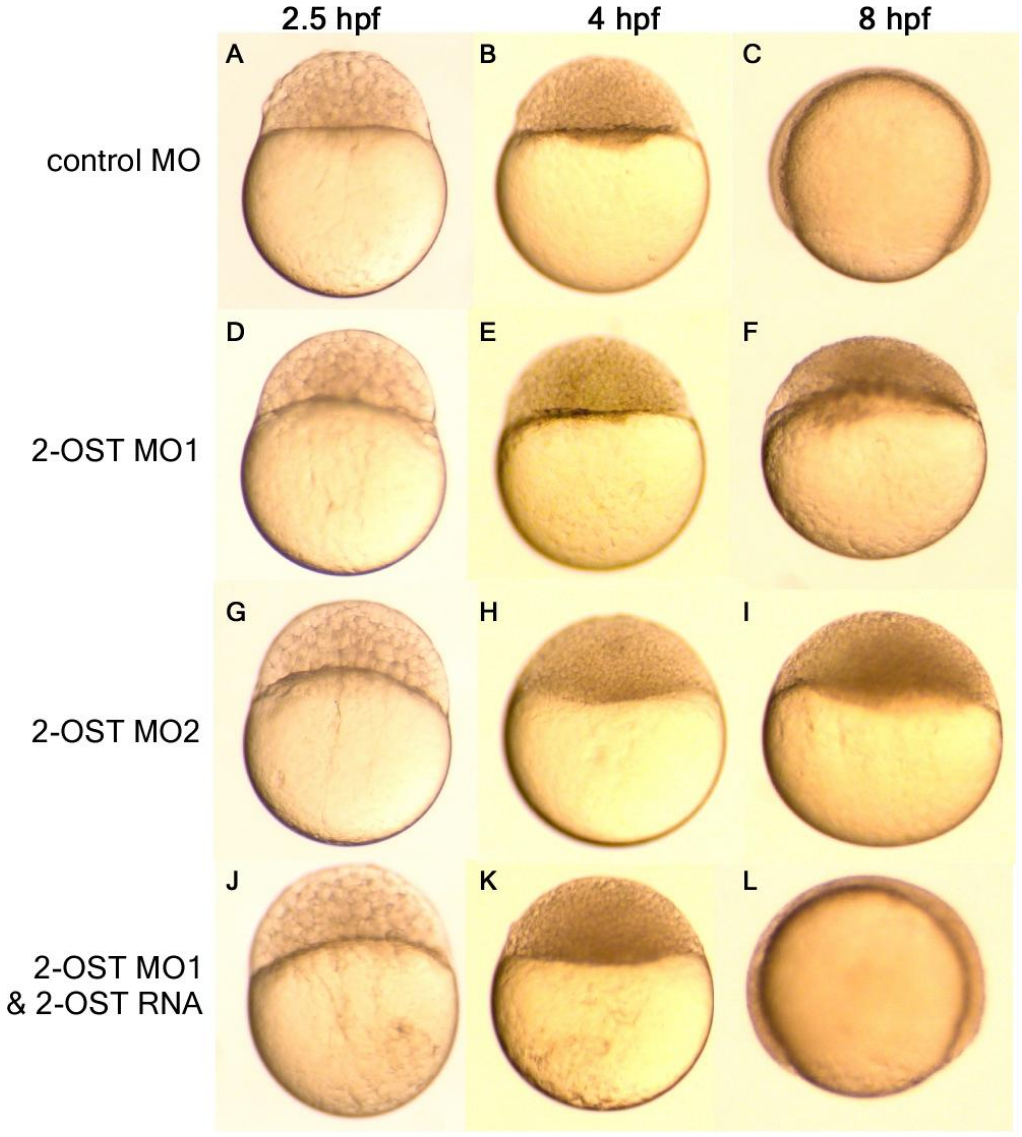
Results

Epiboly Initiation Requires 2-O-Sulfation

To address the roles of 2-OST in development, two different translation blocking (AUG) morpholinos (MO) were injected at the 1-2 cell stage. Blocking both the maternal and zygotic transcript was essential, as 2-OST mRNA is maternally deposited and ubiquitously expressed through gastrulation (Cadwallader and Yost, 2007). As a control, an AUG MO against another ubiquitously expressed GAG chain modifying enzyme, 3-O-sulfotransferase-7, displayed no early embryonic phenotype. The control morphants progressed through the initiation of epiboly and through gastrulation normally (Fig. 2.1A-C). Embryos injected with either 2-OST MO1 or 2-OST MO2 developed normally up to the point of epiboly initiation (Fig. 2.1D,E,G,H). However, at the onset of epiboly, EVL cells failed to initiate migration in the vegetal direction, resulting in cells remaining atop the yolk cell (Fig. 2.1F,I). Eventually 2-OST morphants died between 5 and 10 hpf, apparently by contraction in the yolk at the margin of the blastodisc and lysis of the yolk cell (Supplementary Movie 1).

As an important control for morpholino specificity, the epiboly initiation defect in 2-OST morphants can be rescued by co-injection of mRNA encoding 2-OST protein. Embryos injected with 2-OST MO1 and 200 pg 2-OST mRNA initiate epiboly in parallel with control morphants and proceed through gastrulation, resulting in apparently normal embryos at 24 hpf (Fig. 2.1J-M). Injection of 200 pg of 2-OST mRNA alone caused no apparent overexpression phenotype. The ability to rescue the epiboly phenotype with 2-

Figure 2.1. 2-OST is necessary for epiboly in early zebrafish development. 1-2 cell embryos were injected with 3 ng control MO (A-C), 3 ng of 2OST MO1 (D-F), 3 ng of 2OST MO2 (G-I), or mixture of 3 ng 2OST MO1 and 0.2 ng 2OST RNA (J-L), then incubated in embryo water at 28°C. They were examined at 2.5 hpf (A,D,G,J), 4 hpf (B,E,H,K), and 8 hpf (C,F,I,L). Quantitative comparison of injections and rescue scored based on failure to initiate epiboly (M). The co-injection of 2-OST MO+2-OST RNA ($P<0.05$, indicated by asterisks) rescued the percent of embryos that fail to initiate epiboly in the 2-OST MO alone or the 2-OST MO+control RNA by over 50% .


2.5 hpf
4 hpf
8 hpf
A
B
C
control MO
D
E
F
2-OST MO1
G
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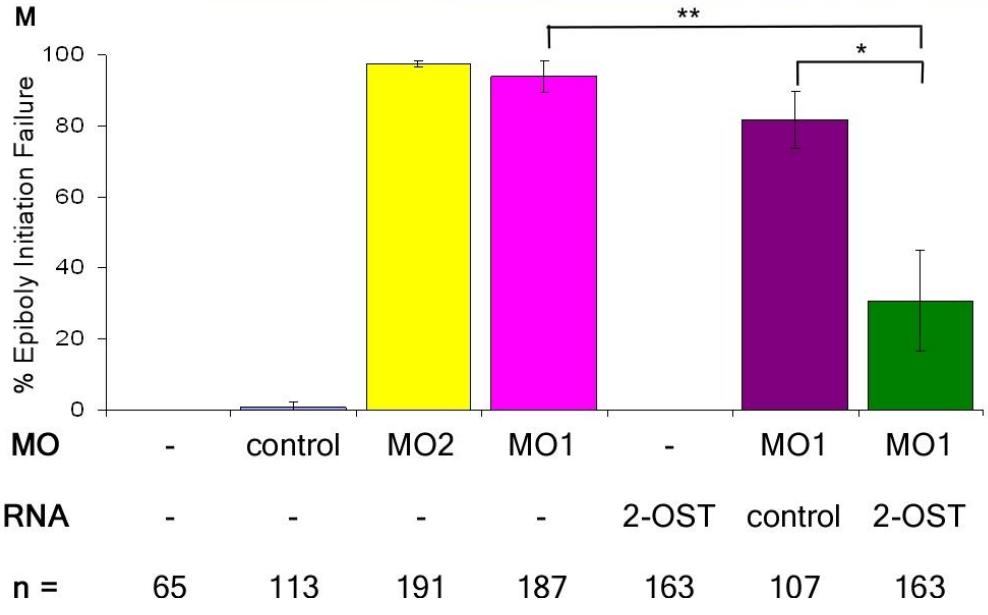
2-OST MO2
J
K
L
2-OST MO1
& 2-OST RNA


M
% Epiboly Initiation Failure
100
80
60
40
20
0
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*
MO - control MO2 MO1 - MO1 MO1
RNA - - - - 2-OST control 2-OST
n = 65 113 191 187 163 107 163

OST mRNA indicates that the morphant phenotypes reflect the knockdown of 2-OST and not off target MO affects.

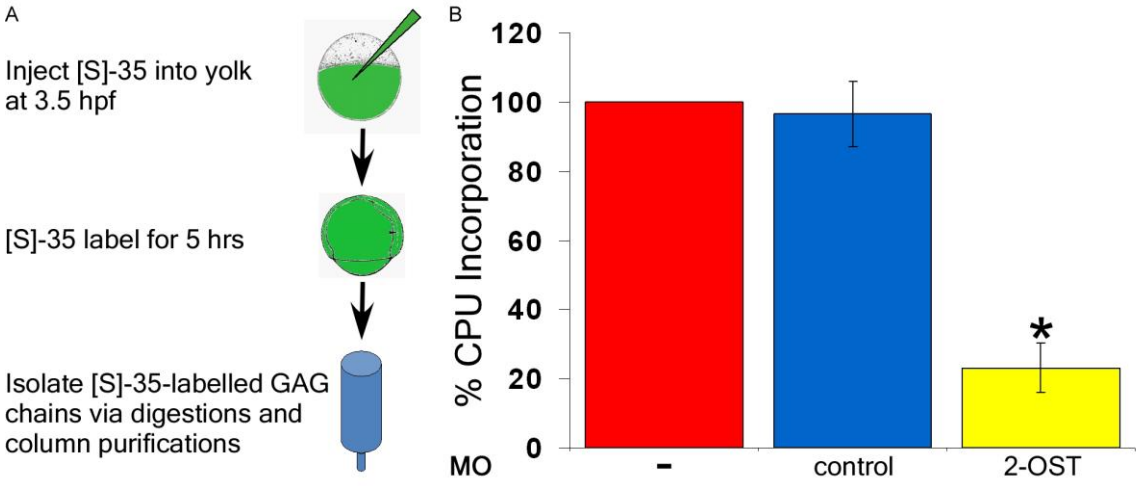
2-O-Sulfation Levels Reduced in 2-OST Morphants

To determine whether morpholino knockdown of 2-OST results in a reduction of the levels of sulfation of GAG chains, WT embryos, control morphants, and 2-OST morphants were pulse-labeled with [S]-35 radionuclide. A variety of radio-labeling methods were tested, including soaking regular and dechorionated embryos in [S]-35, and injection into the chorion or the yolk [S]-35. The most effective approach was found to be injection of [S]-35 directly into the yolk, at approximately 3 hpf and allowing label incorporation for 5 hours (data not shown). Viable embryos were collected at 8 hpf, with equal numbers of embryos from each group (30 embryos/group, n=3). Embryos were digested overnight in Pronase solution and GAG chains were isolated using several purification steps. The levels of [S]-35 incorporation into GAG chains were measured for each sample, amount of radiolabel-incorporation per embryo determined, and levels were normalized to the WT embryos (Fig. 2.2). GAG sulfation was five-fold less in 2-OST morphants compared to WT and control morphants, indicating that 2-OST morpholino was able to knockdown 2-OST activity by 80% during the stages preceding epiboly. These results, in combination with the 2-OST RNA rescue experiments, show that the epiboly initiation defect is due to a decrease in 2-O-sulfation in the embryo.

2-OST Morphants Establish Germ Layers and Form Axes

Gastrulation is a complex process by which the initial body plan for the developing embryo is established. It involves several highly coordinated and carefully regulated cell movements which are performed to allow the developing zebrafish to

Figure 2.2. 2-OST morphants display reduced [S]-35 incorporation. WT, control MO, and 2-OST MO embryos were injected with 1 nL 10mCi [S]-35, grown for 5 hours, and collected at 8 hpf (n=3, 90 embryos group/n). Protein purification was performed to isolate radiolabeled GAG chains and CPM/embryo was calculated based on Liquid Scintillation counting results (A). Ratio of [S]-35 incorporation per embryo was normalized to WT. The 2-OST morphants exhibit a nearly 80% decreased rate of incorporation of [S]-35 ($P<0.05$ indicated by asterisk) compared to WT and control morphants (B).


A
Inject [S]-35 into yolk
at 3.5 hpf
[S]-35 label for 5 hrs
Isolate [S]-35-labelled GAG
chains via digestions and
column purifications
B
% CPU Incorporation
120
100
80
60
40
20
0
*
MO
-
control
2-OST

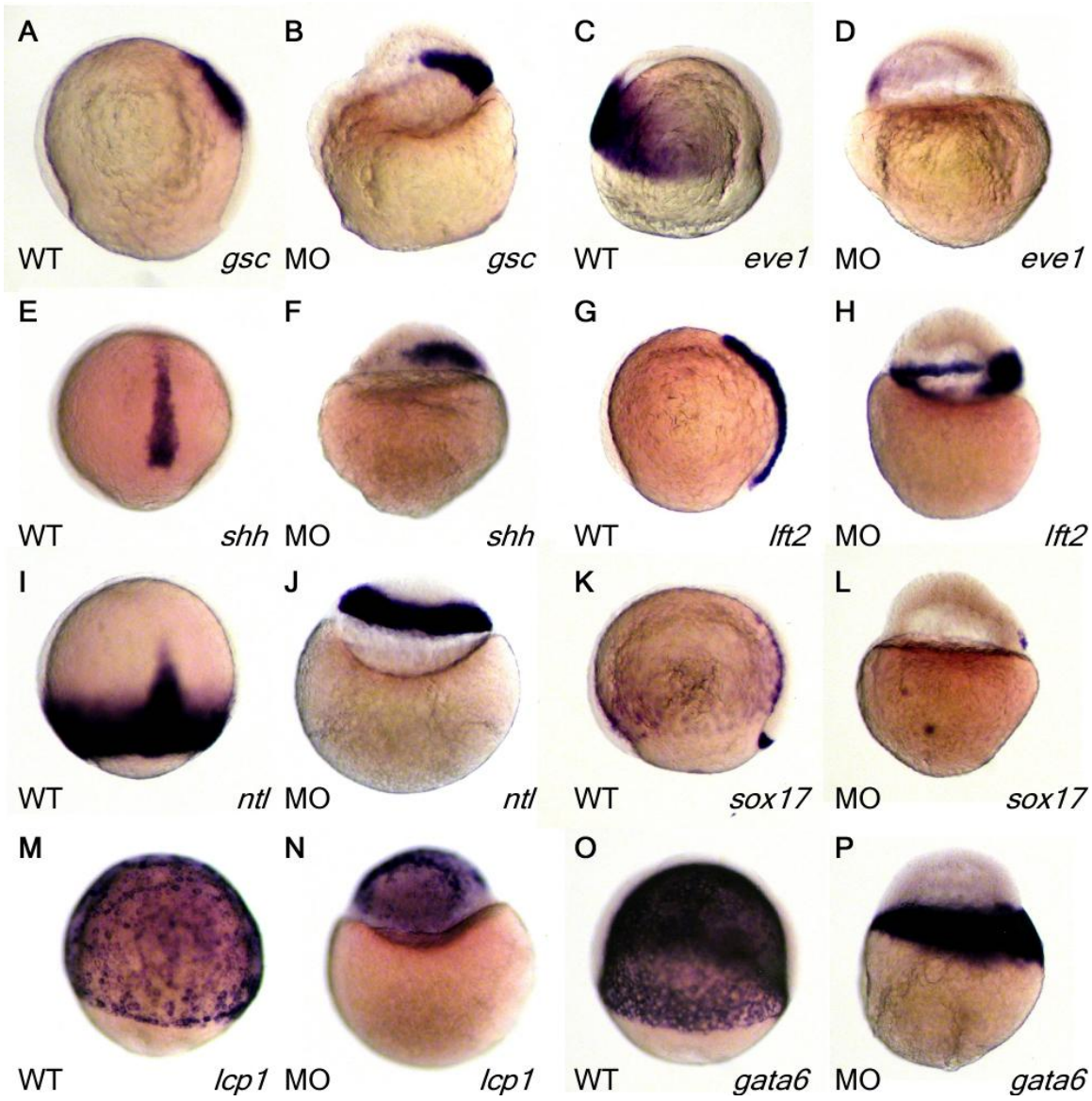
organize cells into primary germ layers distributed along the body axes (Warga and Kimmel, 1990; Rohde and Heisenberg, 2007). To assess whether the epiboly defect caused by the 2-OST MO knockdown is due to a failure to properly develop embryonic axes, *in situ* hybridization was performed at 8 hpf with several patterning markers to determine whether axes were established and primary germ layer precursors were present. We examined mesoderm germ layer markers including *goosecoid* (*gsc*), a dorsal marker (Fig. 2.3A,B), *eve1*, a ventral marker (Fig. 2.3C,D), and *sonic hedgehog* (*shh*), a dorsal midline marker (Fig 2.3E,F). *Lefty2* (*lft2*) and *No tail* (*ntl*) are dorsal and marginal mesendoderm markers, respectively (Fig. 2.3G-J). *Sox17* is a marker for both endoderm as well as the dorsal forerunner cells (DFC) (Fig. 2.3K,L). *Lcp1* marks the EVL endoderm (Fig. 2.3M,N) and *Gata6* marks differentiating yolk syncytial layer (YSL) (Fig. 2.3O,P).

The presence and localization of each of these markers indicates that the 2-OST morphants, although failing to initiate epiboly, establish embryonic axes and germ layer precursors. Transcription of *Shh* and *Sox17* does not begin until midgastrulation, the expression of these markers in 2-OST morphants suggests that development is not significantly delayed. This indicates that the defect in epiboly initiation appears to be a specific cell behavior defect, not a developmental arrest of germ layer specification or subsequent embryonic axis formation.

Knockdown of 2-OST in Zebrafish Embryos Reduces Adhesion Between Deep Cells

Previous studies have supported an active role for deep cells in the initiation of epiboly, with cell intercalations promoting the doming of the blastodisc over the yolk,

Figure 2.3. Molecular marker expression in WT and 2-OST morphant embryos. Wild-type embryos were injected with 3 ng of either control MO or 2OST MO, fixed at 8 hpf, and processed for whole-mount in situ hybridization. Expression patterns of mesoderm markers (A-F). Expression patterns of mesendoderm markers. (K-L) Expression patterns of DFC and endoderm marker (G-J). Expression patterns of ectoderm marker (M,N). Expression patterns of YSN marker (O,P). The presence and organization of these markers in both the WT (n=35) and morphant embryos (n=30) suggest that 2-OST morphants are establishing the primary germ layers and axes. Furthermore, the presence of sox17 and shh, which come on later in development, indicate the 2-OST morphants are not severely delayed in development.

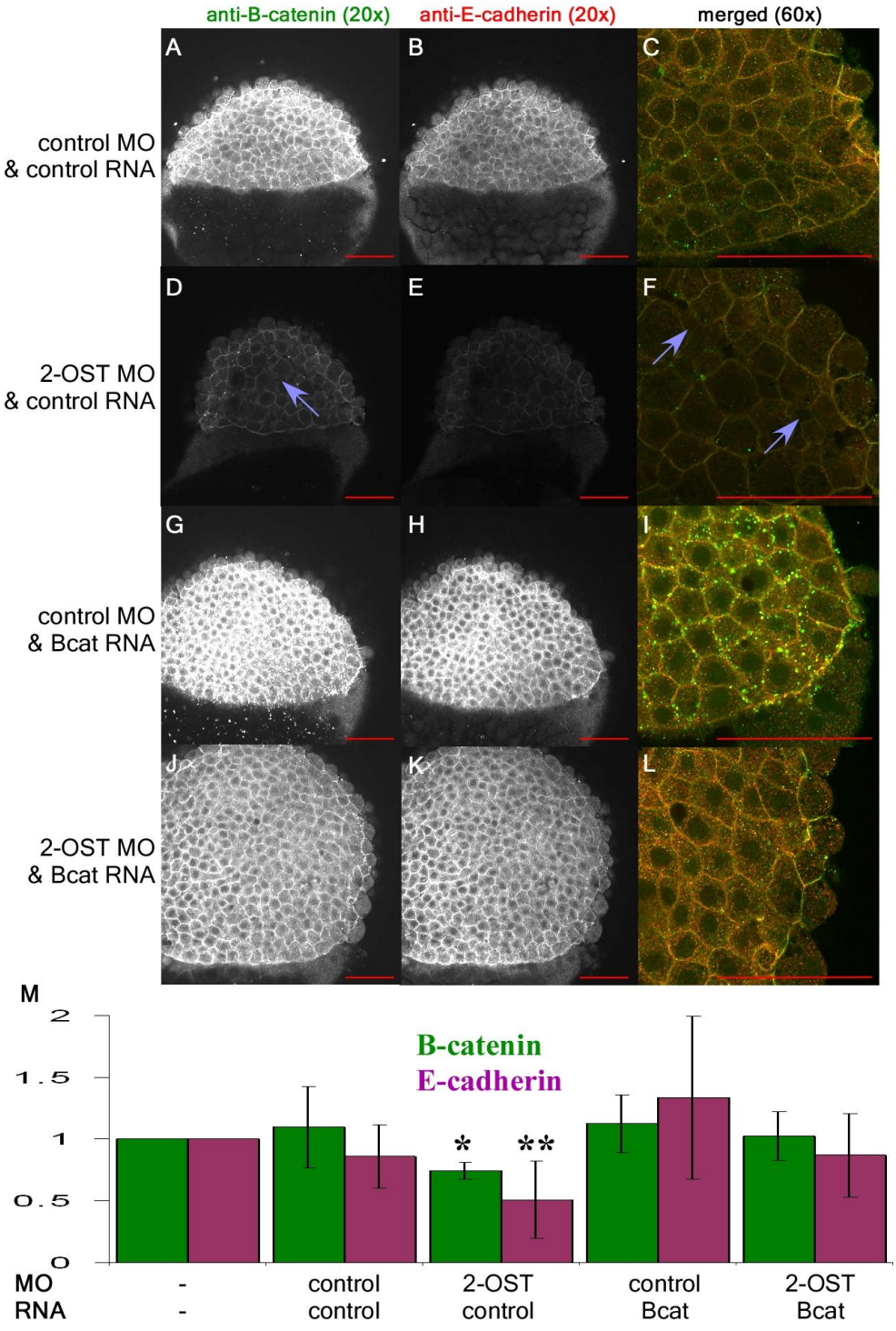

A
WT gsc
B
MO gsc
C
WT eve1
D
MO eve1
E
WT shh
F
MO shh
G
WT lft2
H
MO lft2
I
WT ntl
J
MO ntl
K
WT sox17
L
MO sox17
M
WT lcp1
N
MO lcp1
O
WT gata6
P
MO gata6

and loss of adhesion among deep cells resulting in slowed or failed epiboly (Babb and (Babb and Marrs, 2004; Kane et al., 2005; Adachi et al., 2009). To determine if reduced adhesion between the deep cells contributes to the epiboly defect in 2-OST morphants, we examined the accumulation and intracellular localization of the adherens junction proteins β-catenin and E-cadherin. WT embryos and control morphants had high levels of β-catenin protein localization (Fig. 2.4A,C) and E-cadherin localization (Fig. 2.4B,C) to the cell membranes in the EVL and the deep cells, with cell morphologies that reflect strong evels of adhesion among the deep cells. In contrast, 2-OST morphants had decreased intensity of protein localization of β-catenin (Fig. 2.4D,F) and E-cadherin (Fig. 2.4E,F) to the membranes of both the EVL and deep cells. The 2-OST morphant cell morphology is more rounded, suggesting a lack of tension due to the reduced adhesion. Additionally, gaps were present between the deep cells, suggesting regions where the cells had lost adherence to one another. Furthermore, the levels of β-catenin and E-cadherin protein accumulation were reduced in 2-OST morphants, based on Western blot analyses (Fig. 2.4M). This suggests that reduced adhesion between the 2-OST morphant deep cells might contribute to the failure to initiate epiboly.

2-OST Morphant Adhesion Defects Can Be Rescued by β-catenin

We next addressed whether restoring the levels of β-catenin could rescue the adhesion defects in 2-OST morphants. mRNA encoding a stabilized β-catenin, which cannot be targeted for degradation by the destruction complex (Dorsky et al., 2002), was co-injected with the control MO resulting in increased intensity of the protein levels of both β-catenin (Fig. 2.4G,I) and E-cadherin (Fig. 2.4H,I) at the cell membrane. Similarly,

Figure 2.4. β-catenin accumulation is dependent on 2-OST. Single plane of z-stack of β-catenin for control MO+control RNA (A), 2-OST MO+control RNA (D), control MO+stabilized β-catenin RNA (G), and 2-OST MO+stabilized β-catenin RNA (J) embryos. Single plane of z-stack of E-cadherin control MO+ control RNA (B), 2-OST MO+control RNA (E), control MO +stabilized β-catenin RNA (H), and 2-OST MO+stabilized β-catenin RNA (K) embryos. Merged images of β-catenin and E-cadherin for control MO+control RNA (C), 2-OST MO+control RNA (F), control MO+stabilized β-catenin RNA (I), and 2-OST MO +stabilized β-catenin RNA (L) embryos (n=21 for each group). The purple arrows indicate regions where adhesion is completely lost between deep cells. Scale bars are 100 um. Western results comparing levels of β-catenin normalized to α-tubulin (green) and E-cadherin normalized to α-tubulin (red) for WT, control MO and control RNA, 2-OST MO and control RNA, control MO and stabilized β-catenin RNA, and 2-OST MO and stabilized β-catenin RNA, each group normalized to WT (M) (n=4 embryos/lane/sample, experiments were repeated in triplicates).


anti-B-catenin (20x)
anti-E-cadherin (20x)
merged (60x)
A
B
C
control MO
& control RNA
D
E
F
2-OST MO
& control RNA
G
H
I
control MO
& Bcat RNA
J
K
L
2-OST MO
& Bcat RNA
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2
1.5
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B-catenin
E-cadherin
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2-OST
Bcat

2-OST morphants co-injected with the stabilized β-catenin mRNA had increased levels of β-catenin (Fig. 2.4J,L) and E-cadherin (Fig. 2.4K,L) at the cell membrane. The increased levels of β-catenin and E-cadherin in both the control morphants and 2-OST morphants were confirmed by Western analysis (Fig. 2.4M). Strikingly, the β-catenin mRNA injection in 2-OST morphants abolished the gaps between the deep cells, rescued cell morphology and rescued the adhesion defects between the deep cells. However, while the control morphants with injected β-catenin initiated epiboly as expected, the β-catenin rescued 2-OST morphants failed to initiate epiboly.

2-OST Morphants Exhibit Reduced Rates of Proliferation that Can Be Rescued by β-catenin

In addition to its role in modulating adhesion, β-catenin contributes to the transcriptional regulation of genes which regulate cell proliferation (Bienz, 2005; Brembeck et al., 2006). Cells are actively dividing at blastula stages of development and disruptions in proliferation can result in epiboly defects (Kimmel et al., 1995; Rozario et al., 2009). To address whether reduced levels of β-catenin in 2-OST morphants were also affecting this classical aspect of β-catenin signaling, we measured the rates of cell proliferation by employing a BrdU incorporation assay. To determine the rate of proliferation, the total number of nuclei, indicated by the nuclear stain Sytox Green in a cross section of the embryo (Fig. 2.5A,E), was compared to the number of cells which incorporated BrdU over a 20-minute period of time (Fig. 2.5C,G,K). The WT embryos and control morphants incorporated BrdU at the same rate, while only half the number of nuclei in 2-OST morphants had incorporated BrdU over the same time period (Fig. 2.5K).

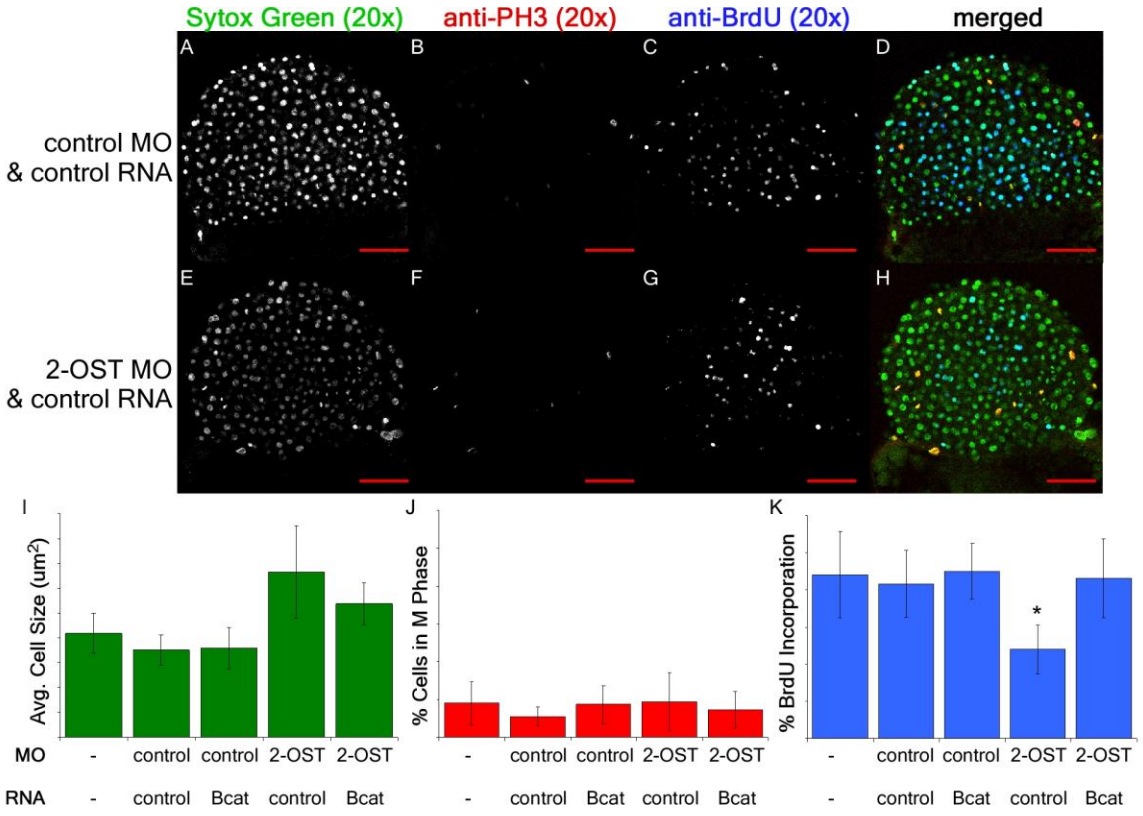
Since cell division in zebrafish embryos results in smaller cell volumes (Kimmel and Law, 1985b), if the rate of proliferation is reduced, the volumes of individual cells are predicted to be larger. Therefore, as a control study, average cell area was calculated from the total area of each embryo cross section and the number of nuclei in that section. The average cross sectional cell area of 2-OST morphants was larger than the control embryos (Fig. 2.5I). To test whether the 2-OST morphants were stalled during mitosis an antibody against phospho-histone-3 (PH3; Fig. 2.5B,F,J) was used as a marker of M phase to determine the percentage of cells in M phase at any given time. The differences between the WT, control, and 2-OST morphant embryos were not statistically significant (Fig. 2.5J).

We next sought to determine whether these cell cycle defects were dependent on altered β-catenin levels, by assessing whether co-injection of the stabilized β-catenin could rescue the cell proliferation defect. Co-injection of the stabilized β-catenin in 2-OST morphants significantly increased the cell proliferation rate, rescuing the 2-OST morphants back to normal cell proliferation rates (Fig. 2.5K). Stabilized β-catenin did not alter the normal proliferation rate in the control morphants (Fig. 2.5K). Together, results from cell morphology and cell cycle studies suggest that 2-OST acts upstream of β-catenin function.

2-OST Modulates Wnt Signaling Upstream of GSK3 and Downstream of Wnt8

To examine if the 2-OST-dependent regulation of the accumulation and cellular functions of β-catenin is dependent on the canonical Wnt cell signaling pathway, epistasis experiments were performed to test whether components of the Wnt pathway had effects

Figure 2.5. 2-OST contributes to regulation of cell proliferation via β-catenin. Single plane of z-stack of Sytox Green nuclear stain for control MO+control RNA (A) and 2-OST MO+control RNA embryos (E). Anti-phopho-histone H3 for control MO+control RNA (B) and 2-OST MO+control RNA embryos (F). Anti-BrdU for control MO+control RNA (C) and 2-OST MO+control RNA embryos (G). Merged images of sytox green, anti-phospho-histone-H3, and anti-BrdU for control MO+control RNA (D) and 2-OST MO+control RNA embryos (H). Scale bars are 100 um. Chart comparing average cell area (I). Chart comparing percent of cells in M phase (J). Chart comparing rate of cell proliferation (K). (n=15 per group). The rate of proliferation in the 2-OST morphants (asterisk indicates $P<0.05$) was nearly half that observed in WT and control morphants. The 2-OST+β-catenin RNA rescued the proliferation defect seen in the 2-OST morphants ($p<.127$).


Sytox Green (20x)
anti-PH3 (20x)
anti-BrdU (20x)
merged
A
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D
control MO
& control RNA
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2-OST MO
& control RNA
I
Avg. Cell Size (um2)
J
% Cells in M Phase
K
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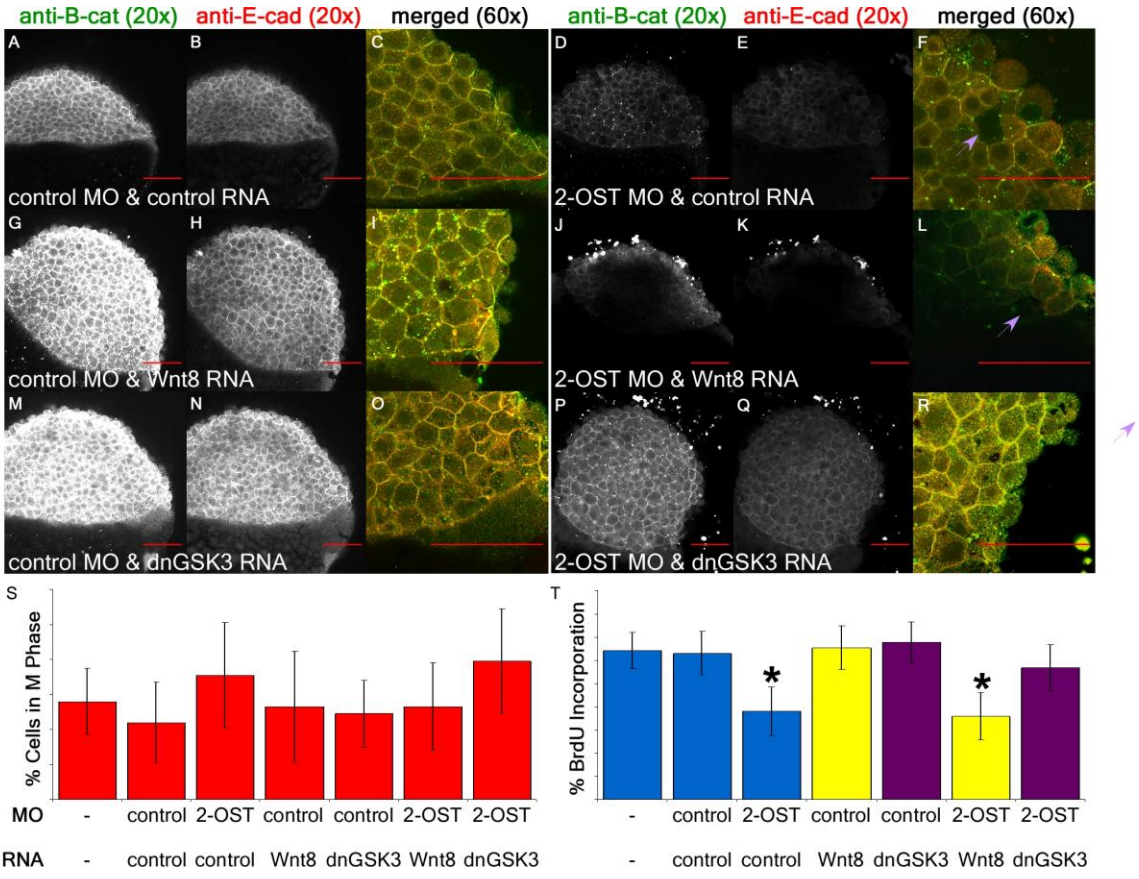
MO - control control 2-OST 2-OST
RNA - control Bcat control Bcat

on 2-OST morphants (Kelly et al., 1995). Wnt8 is a ligand which binds cell surface receptors, initiating the canonical Wnt signaling pathway. GSK3 is a part of the intracellular destruction complex which promotes the degradation of β-catenin in the absence of Wnt signaling. Expressing a dominant negative GSK3 (dnGSK3) protein interferes with destruction complex, preventing the degradation of β-catenin and thus stabilizing its intracellular levels and simulating Wnt signaling.

The ectopic expression of Wnt8 increased the levels of β-catenin and E-cadherin in control morphants (Fig. 2.6G,H) over those in embryos co-injected with control RNA injected and control MO (Fig. 2.6A,B). This indicates that Wnt8 is capable of activating the downstream Wnt response pathway in embryonic deep cells. Similarly, the expression of a dnGSK3 protein also increased the levels of β-catenin and E-cadherin in control morphants (Fig. 2.6J,K), suggesting that this component of the intracellular Wnt signaling pathway is responsive in embryonic deep cells. Strikingly, the ability of Wnt8 to increase the levels of β-catenin and E-cadherin was blocked in 2-OST morphants. Wnt8 overexpression did not rescue the adhesion defects (Fig. 2.6M,N) and the proliferation defects (Fig. 2.6S) in the embryonic deep cells of 2-OST morphants. In contrast, co-injection of dnGSK3 in 2-OST morphants rescued β-catenin and E-cadherin protein levels (Fig. 2.6P,Q), and partially rescued of the proliferation defects (Fig. 2.6T). No significant difference was observed between the different populations in terms of the percent of cells in M phase (Fig. 2.6S). Together, the results of these epistasis experiments suggest that 2-OST functions downstream of the activity of the Wnt ligands but upstream of the destruction complex for β-catenin.

Figure 2.6. 2-OST modulates Wnt signaling upstream of GSK3 and downstream of Wnt8. Single plane of z-stack of β-catenin for control MO+control RNA (A), 2-OST MO+control RNA (D), control MO+Wnt8 RNA (G), 2-OST MO+Wnt8 RNA (J), control MO+dnGSK3 RNA (M), and 2-OST MO+dnGSK3 RNA (P). Cross section of E-cadherin for control MO+control RNA (B), 2-OST MO+control RNA (E), control MO+Wnt8 RNA (H), 2-OST MO+Wnt8 RNA (K), control MO+dnGSK3 RNA (N), and 2-OST MO+dnGSK3 RNA (Q). Merged and magnified cross section of β-catenin and E-cadherin for control MO and control RNA (C), 2-OST MO+control RNA (F), control MO+Wnt8 RNA (I), 2-OST MO+Wnt8 RNA (L), control MO+dnGSK3 RNA (O), and 2-OST MO+dnGSK3 RNA (R). Scale bars are 100 um. Chart comparing percent of cells in M phase (S). Chart comparing rate of proliferation (T). (n=15 for each group) The rate of proliferation for the 2-OST MO+control RNA and 2-OST MO+Wnt8 RNA (asterisks indicate $p<<0.05$) is nearly half the rate of the controls. However, there is a considerable rate of rescue in the 2-OST MO+dnGSK3 embryos ($p<0.02$) even though it is not a complete rescue.


anti-B-cat (20x)
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Discussion

The manner by which components of the extracellular matrix impact cell behavior has been the focus of research for some time. However, HSPGs are a relatively new branch of this field of study. Studies in cell culture and invertebrates have begun to establish the range and complexity of signaling cascades regulated by these cell surface proteoglycans. However, not much work has been done so far to understand the role these compounds play in early vertebrate development. This study reveals the roles of 2-O-sulfation on HSPG GAG chains in vertebrate development, which is required for Wnt-pathway-dependent cell adhesion and proliferation of embryonic deep cells and the initiation of epiboly in zebrafish. Reducing the function of 2-OST resulted in an embryonic lethal phenotype caused by uncoupling cell adhesion and cell proliferation from the unperturbed processes of germ-layer specification and embryonic patterning.

The roles for β-catenin in vertebrate epiboly have not been explored. β-catenin interacts with E-cadherin to stabilize the adherens junctions that contribute to adhesive forces (Perez-Moreno et al., 2003; Perez-Moreno and Fuchs, 2006; Lecuit and Lenne, 2007; Hammerschmidt and Wedlich, 2008). Here we show that endogenous β-catenin protein levels are reduced in 2-OST morphants suggesting that the cell adhesion and cell proliferation defects in 2-OST morphants are due to a combined reduction of β-catenin and E-cadherin at the cell junctions in deep embryonic cells. E-cadherin has previously been demonstrated to play a crucial role in the epiboly of the deep cells (Babb and Marrs, 2004; Shimizu et al., 2005). Our examination of protein levels revealed a reduction in E-cadherin membrane localization, resulting in reduced adhesion between the deep cells to themselves as well as between the EVL and the deep cells. This last interaction is key for

the deep cells to fill in between the yolk and EVL during epiboly. Reduced membrane localization and the reduced total level of both these proteins suggest adhesion may be mediated by 2-OST activity and furthermore that 2-OST may play a role in supporting Wnt signal transduction.

To examine another facet of β-catenin signaling mediated by Wnt signaling we assessed the rates of cell proliferation in the 2-OST morphants compared to controls (Nelson and Nusse, 2004). The rate of cellular proliferation, based on the incorporation of BrdU over a finite period of time, for 2-OST morphants was nearly half that of the controls, with a corresponding increase in cell area. The 2-OST morphants did not display an increased level of Histone H3 phosphorylation, suggesting they were not slowing or stalling in M phase. This suggests 2-OST morphants were slowing at another part of the cell cycle.

β-catenin regulates transcription of Cyclin D1, a key part of the G1 checkpoint in cell cycle regulation (Tetsu and McCormick, 1999), and therefore it is possible that modifications to 2-O-sulfation result in reduced β-catenin levels, reducing the level of Cyclin D1 which resulted in the slower rate of cell proliferation in the morphants. However, the regulation of cell cycle progression is a complex network of signaling cascades and Cyclin D1 is subject to regulation by a variety of factors so other explanations are also possible.

The reduced adhesion based on decreased levels of E-cadherin and β-catenin and decreased proliferation rates in the 2-OST morphants suggested manipulation of these proteins might rescue these defects. Co-expression of a stabilized form of β-catenin rescued the adhesion defects and cell membrane localization of both β-catenin and E-

cadherin in 2-OST morphants. To determine if 2-OST modifications modulate β-catenin via the canonical Wnt signaling epistasis experiments were conducted to look at an extracellular and an intracellular element of the cascade, both of which are upstream of β-catenin activity. Expression of the dnGSK3 interferes with the intracellular β-catenin destruction complex, resulting in an increased pool of β-catenin, which in both the control and 2-OST morphants increased localization of β-catenin and E-cadherin to the cell membrane. This rescues the 2-OST morphant deep cell adhesion defects and also partially rescues the proliferation defects in the 2-OST morphants. However, expression of Wnt8, a secreted extracellular ligand which activates the canonical Wnt cascade increasing levels of β-catenin in control embryos, fails to increase cell membrane localization of β-catenin or E-cadherin, rescuing neither the cell adhesion defects nor the cell proliferation defects in 2-OST morphants.

Although reactivating the canonical Wnt cascade rescues the defects in proliferation and adhesion in an epistatic fashion downstream of where 2-OST activity is manifested, it fails to rescue the epiboly initiation defect. This is likely because the HS GAG modifications driven by 2-OST control not only Wnt signaling pathways but other pathways as well, predicting that the epiboly defect is a compound phenotype of defective Wnt-pathway-dependent cell adhesion and proliferation of embryonic deep cells, as well as Wnt-independent pathways that would not be rescued by stabilization of the β-catenin protein levels. This is in keeping with the observation that knockdown of β-catenin in zebrafish results in later developmental defects (Bellipanni et al., 2006). The process of epiboly is controlled by several different components of the cytoskeleton in the yolk in addition to the intercalation and spreading of embryonic deep cells

manipulated here (Strahle and Jesuthasan, 1993; Solnica-Krezel and Driever, 1994; Zalik et al., 1999; Cheng et al., 2004). However, the genes which regulate these processes are only beginning to be identified and understood in a context dependent set of signaling cascades.

One possible explanation for why the morphants fail to initiate epiboly is that the gradient of E-cadherin expression is altered in both the morphant as well as the β-catenin rescued embryos. One theory for how the deep cells contribute to epiboly stems from the differential adhesion hypothesis initially put forth by Townes and Holtfreter. In a series of elegant studies they demonstrated that cells spontaneously sort based on selective adhesion, cells exhibit the greatest affinity for other cells expressing cell surface markers most similar to their own profile which allows them to establish the most energetically favorable arrangement (Townes and Holtfreter, 1955). In situ hybridization cross-sections for E-cadherin reveal that a gradient of expression is present in the blastodisc during gastrulation. *Cdh1* levels are highest in the EVL and decrease as the cell layers become less superficial (Kane et al., 2005). Studies suggest that in normal development the deep cells and EVL become thin and spread out as the less superficial deep cells intercalate with the more superficial cells of the blastodisc deep cell population. When E-cadherin levels are disrupted however, this process is disrupted. Therefore, it is possible that due to the decreased levels of E-cadherin in the 2-OST morphants, the cells fail to interact properly to promote epiboly. Furthermore in 2-OST morphants rescued with β-catenin, despite E-cadherin localization to the cell surface, the correct gradient of expression may not be established, preventing the embryos from initiating epiboly properly as well.

Another explanation is that although the E-cadherin localization was rescued, the functionality was not. This is perhaps due to reduced levels of extracellular calcium, which is needed for both E-cadherin function and epiboly (Webb and Miller, 2006). Other molecules play a role in this interaction and it is possible their levels remain diminished in spite of the rescue, so increased levels of E-cadherin/β-catenin might not be sufficient. Based on the rescue of the protein levels by western blot, it also suggests that β-catenin contributes to the regulation of E-cadherin accumulation. These results suggest other pathways are also required in the 2-OST-dependent initiation of epiboly which will be explored in a future study.

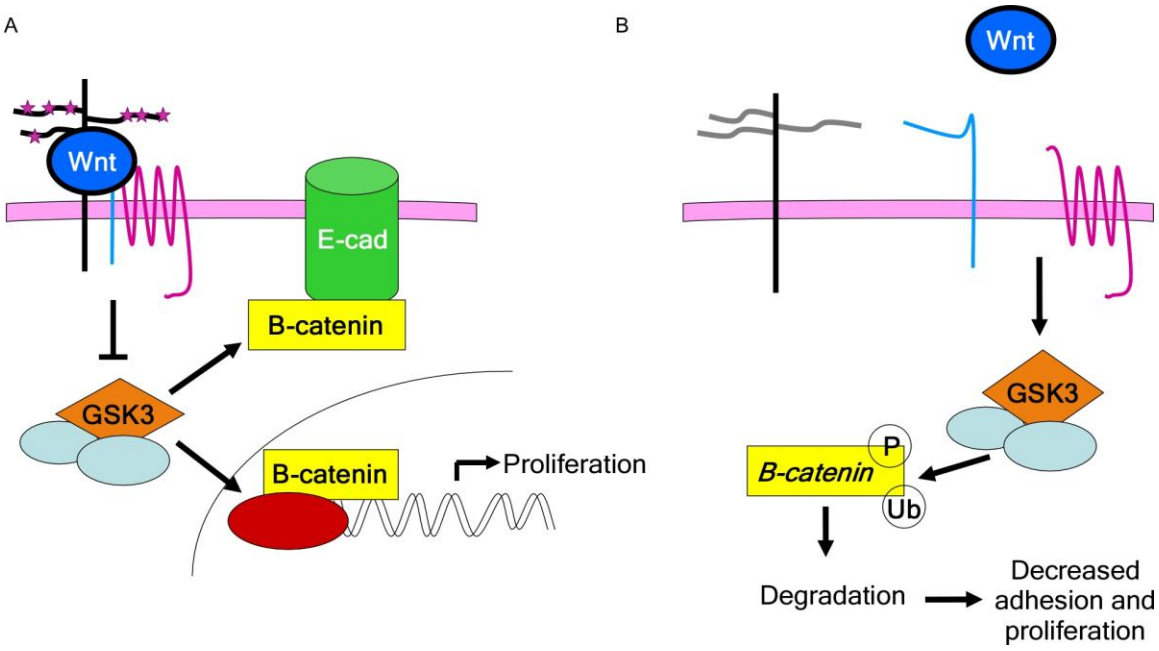
From these sets of experiments we conclude that reduced 2-O-sulfation prevents full activation of the Wnt signaling cascade in morphant embryos (Fig. 2.7). However, as rescue of this signaling cascade fails to rescue any part of the epiboly initiation defect, we conclude that other pathways are also affected by this loss of sulfation due to a compound phenotype, analogous to the multiple signaling pathways in Drosophila that are HS-dependent. Future studies will address roles other possible signaling cascades may play in regulating epiboly and how the absence of 2-O-sulfation is modifying them.

Materials and Methods

Zebrafish Lines

Oregon AB wild-type zebrafish (Danio rerio) were maintained on a 14-hour light/10-hour dark cycle at 28.5 degrees Celsius. Zebrafish embryos resulted from natural spawning and were collected, injected, raised, and staged as previously described (Essner et al., 2005).

Figure 2.7. Model for role of 2-O-Sulfation in canonical Wnt signaling cascade. (A) Canonical Wnt signaling in the presence of 2-O-sulfation. (B) Wnt signaling in the absence of 2-O-sulfation.


A
Wnt
E-cad
B-catenin
GSK3
B-catenin
Proliferation
B
Wnt
GSK3
P
B-catenin
Ub
Degradation
Decreased
adhesion and
proliferation

Morpholino and RNA Injection of Zebrafish Embryos

The following morpholino oligonucleotides were used: 2-OST MO1 (5'-TGACCGAGAACTTTATTACACACAG -3'), 2-OST MO2 (5'-AAGCCCCATCAAAAAATCCAGCAGG-3'), and as a control 3-OST-7 MO (5'-CACATAACTCAGAAGATTGGCCATG -3'). The first 2-OST specific MO (2-OST MO1) targeted the sequence directly upstream of the transcription initiation site. The second 2-OST specific MO (2-OST MO2) recognized the region surrounding the translation start site, not overlapping the first MO. Embryos were injected at the 1-2 cell stage. For the rescue of the 2-OST MO1 phenotype, 200 pg of 2-OST mRNA (GenBank Accession number DQ812997.1) was co-injected with the morpholino. B-galactosidase mRNA was injected as a control (GenBank Accession number AP_000996). The 2-OST mRNA was cloned out of the pBSII T7 vector and into a modified pCS2+ expression vector using the *Nco1* and *Xba1* restriction enzymes. pCS2+-stabilized β-catenin and pT7T-Wnt8 expression vectors were gifts from Rich Dorsky. The Xenopus pCS2+-dnGSK3 expression vector was a gift from Monica Vetter. RNA was made using respective Message Machine kits (Ambion).

[S]-35 Radiolabeling

Embryos were injected with morpholino at 1-2 cell stage. At 3.5 hpf [S]-35 was injected into the yolk. The embryos were cultured at room temperature for 5 hours. Only viable embryos were then collected, 30 embryos per group, in PBS and Pronase solution, and incubated overnight. GAG chains were purified in a series of steps requiring 0.2 uM filtration, DEAE column filtration, and PD10 desalting columns (Lawrence et al., 2008).

Liquid Scintillation Counter was used to determine the rate of incorporation for each sample.

In Situ Hybridization

For in situ hybridization analysis embryos were fixed overnight at 4 degrees in sucrose buffered 4% Paraformaldehyde. They were subsequently rinsed in phosphate buffered saline (PBS), dehydrated in methanol, and stored at -20 degrees. Clones encoding the open reading frame (ORF) of *goosecoid*, *sonic hedgehog*, *eve1*, *sox17*, *notail*, *lefty2*, *lcp1* (a gift from Mike Redd), and *gata6* were used to make probe via in vitro transcription. Antisense RNA was made from those linearized plasmids using Digoxigenin (DIG) RNA Labeling Kit (Roche, IN). In situ hybridizations were carried out according to a previously established protocol (Thisse et al., 1993) using a Biolane HTI in situ machine (Huller and Huttner AG, Tübingen, Germany). Embryos were cleared in 70% glycerol in PBST and photographed using with a Nikon SMZ1000 on a Leica MZ12 dissecting microscope. Digital images were processed using Adobe Photoshop and ACD systems Canvas.

Immunohistochemistry

Immunohistochemical analysis for proteins was performed on embryos fixed overnight at room temperature in 4% paraformaldehyde. They were subsequently rinsed in PBST, dechorionated, and blocked for 1 hour in blocking solution (1% DMSO, 5% goat serum, .5% Triton-X in PBS). They were blocked overnight in parallel using aliquots from a primary antibody in block solution. They were then washed with block solution and subsequently incubated overnight in secondary antibody. The following primary antibodies and dilutions were used: anti-rabbit anti-β-catenin (Sigma, 1:300),

anti-mouse anti-E-cadherin (BD Bioscience, 1:100). Alexa 647 goat anti-mouse was used as a secondary antibody at a 1:200 dilution (Invitrogen). Embryos were mounted in Slow Fade on cover slips and visualized using Olympus FV300 XY. Images were analyzed using ImageJ software. The 20x image intensity levels were unmodified. The brightness and contrast of 60x images are increased in 2-OST morphants to show highlight morphological differences.

Western Blot

Injected embryos were collected in lysis buffer and Western blots were performed as previously described (Link et al., 2006). Primary antibodies used were mouse anti-α-tubulin (AbCam, 1:7500) as a control, rabbit anti-β-catenin (Sigma, 1:2000), and mouse anti-E-cadherin (BD Bioscience, 1:200). Secondary antibodies used were HRP-conjugated goat anti-mouse and HRP-conjugated goat anti-rabbit (Jackson ImmunoResearch, 1:2000). Membranes were exposed using ECL+ Western Blotting Detection System from Amersham. Densitometry analysis was performed using AlphaEase FC.

Statistics

Data were analyzed using a two-tailed Student's *t*-test. Analysis was done by R-Commander software package within the R Statistical Software platform. Results are considered significant when $P<0.05$ and results are expressed as mean ± s.e.m.

Supplemental Movie

Movie shows wild-type embryo on left and 2-OST morphant embryo on right. Time lapse ranges from 4 hpf to 10 hpf.

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CHAPTER 3

2-O-SULFOTRANSFERASE REGULATES YOLK CYTOSKELETAL ELEMENTS AND FGF SIGNALING DURING EPIBOLY IN ZEBRAFISH

Abstract

Epiboly is the process in the developing zebrafish embryo by which the cells of the blastodisc move vegetally from the animal pole to envelop the yolk cell. This complicated process involves a range of cell behaviors and cytoskeletal remodeling within cells that are just beginning to be understood. 2-O-sulfotransferase (2-OST) is an enzyme which catalyzes the transfer of a sulfate group onto glycoscaminoglycan chains (GAG) of heparan sulfate proteoglycans (HSPG). Modifying the sulfation pattern of these GAG chains affects the cell behaviors and cytoskeletal dynamics underlying epiboly. Knocking down 2-OST results in a failure to initiate and progress through epiboly due to alterations in assembly of filamentous actin, in microtubule organization, and in yolk cell endocytosis. Knocking down 2-OST also affects FGF signaling, with reduced 2-O-sulfation promoting FGF8-induced activation of the FGF signaling cascade as measured by levels of phosphorylated ERK. Finally, synchronized knockdown of Syndecans 2 and 4, HSPGs that require 2-OST function for normal sulfation, result in epiboly initiation and cytoskeletal defects that resemble 2-OST morphants. This suggests an HSPG-based mechanism is imperative for early development.

Introduction

Heparan sulfate proteoglycans (HSPGs) are a diverse family of glycoproteins. HSPGs contain glycosaminoglycan (GAG) chains of alternating disaccharide repeats of glucuronic acid and N-acetyl glucosamine (Esko and Selleck, 2002; Kramer and Yost, 2003). The HSPG family includes the transmembrane syndecans, GPI-anchored glypicans, and members not bound directly to the cell membrane including agrins and perlecans (Hacker et al., 2005; Lamanna et al., 2007). GAG chains can be modified by a variety of enzymes which epimerize, sulfate, and de-acetylate them to create complex and highly variable regions of charge density thought to modulate the activity of a wide range of interactions between ligands and receptors (Harmer, 2006).

HSPGs modulate a wide range of cellular behaviors *in vitro* as well as *in vivo* including gastrulation during early zebrafish development (Ringvall et al., 2000; Walsh and Stainier, 2001; Gotte et al., 2008; Reijmers et al.). Gastrulation is the developmental process by which the initial axes and germ layers of the embryo are established. One step in this process is epiboly during which the blastodisc moves and expands vegetally to encapsulate the yolk.

Epiboly occurs through a carefully orchestrated series of cell movements (Kimmel et al., 1995). At roughly 2.5 hours post fertilization (hpf) cells at the margin fuse with the yolk and extrude their cellular contents to form a multinuclear syncytium referred to at the yolk syncytial layer (YSL) (Kimmel and Law, 1985a). The YSL contains nuclei, termed the yolk syncytial nuclei or YSN, that are connected by a cortical array of microtubules extending around the yolk. These microtubules, along with tight junctions between the EVL and yolk, connect the cells of the blastodisc to the yolk and

the YSN to each other (Solnica-Krezel and Driever, 1994). Microtubules promote epiboly by extending to the vegetal pole and pulling down the cells of the blastodisc as they are shortened by uncoupling at the vegetal pole.

As epiboly begins, the most superficial cells of the blastodisc, known as the enveloping layer (EVL), begin to flatten out. The deep cells beneath the EVL begin to intercalate and expanding vegetally in the space between the EVL and yolk (Kane et al., 2005). Endocytosis of the yolk membrane is thought to help reduce the amount of surface area over which the EVL must travel, while promoting the animalwards doming of the yolk up under the expanding blastodisc (Solnica-Krezel and Driever, 1994). Once the margin of the EVL reaches the equator of the yolk, a drawstring of actin forms and constricts to help draw the EVL vegetally, eventually completely enclosing the yolk (Zalik et al., 1999; Cheng et al., 2004). Epiboly ends when the blastopore at the vegetal pole completely closes (Kane and Adams, 2002). Interfering with any one of these processes (cell flattening, expansion of the EVL, intercalation of deep cells, endocytosis of yolk membrane, formation of the actin drawstring) delays or arrests epiboly (Strahle and Jesuthasan, 1993; Solnica-Krezel and Driever, 1994; Zalik et al., 1999; Cheng et al., 2004).

2-O-sulfotransferase (2-OST) is a GAG-chain modifying enzyme which transfers a sulfate group from a phosphate donor, 3'-phosphoadenosine 5'-phosphosulfate (PAPS), to the second carbon position on glucuronic acid. Reducing 2-OST activity by morpholino (MO) blocks sulfation at this position and results in a failure to initiate epiboly. This epiboly initiation defect could be rescued by co-injection of 2-OST RNA to which the MO could not bind. We demonstrated the specificity of this morpholino by

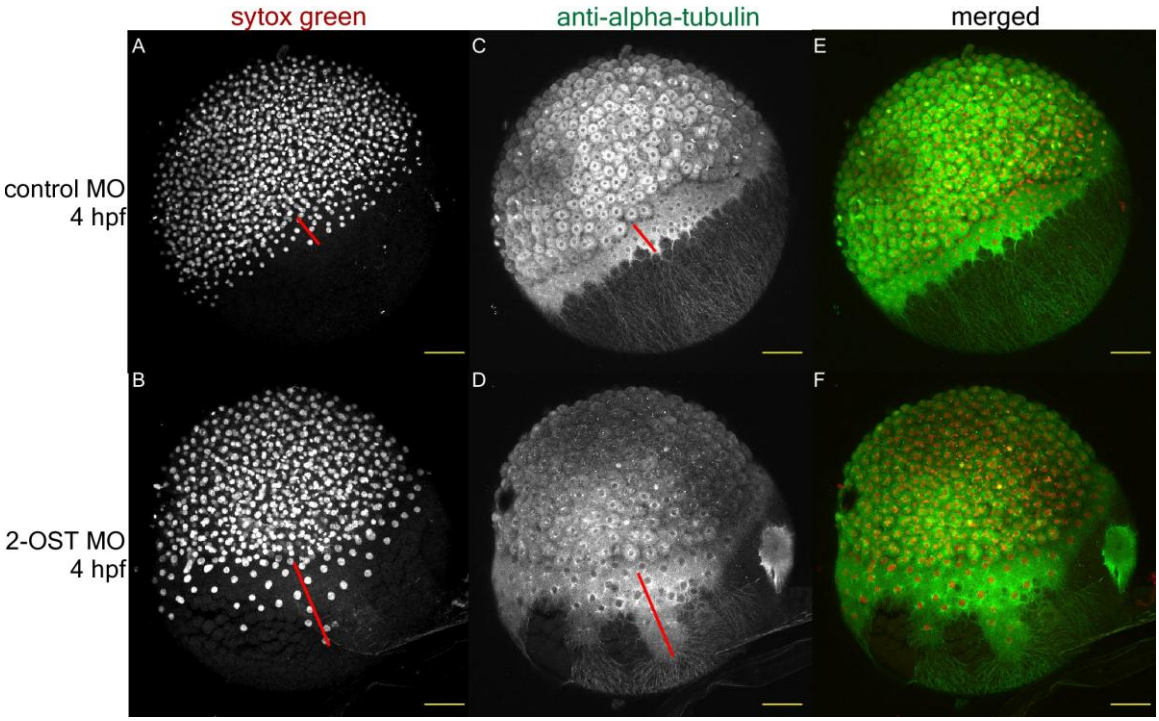
pulse-labeling experiments that showed a statistically significant reduction of incorporation of [S]-35 into the 2-OST MO GAG chains compared to controls (see Chapter 2). In this previous study we focused on activity in the blastodisc contributing to epiboly, demonstrating that 2-OST regulates cell adhesion and proliferation *via* the canonical Wnt cascade. In this study, the focus is largely upon classical components of epibolic regulation in the yolk. We demonstrate that 2-OST knockdown creates a compound phenotype because it also modulates FGF signaling. Furthermore, we have begun to examine candidate molecules that may mediate the effects of 2-OST knockdown, and uncovered an important role for syndecans.

Results

2-OST Controls Organization and Bundling of Microtubules in the Yolk Syncytial Layer

A crucial step for proper epiboly initiation occurs with the formation of the YSL after the marginal cells fuse with and extrude their contents into the yolk (Kimmel and Law, 1985a). Sytox green was used to label nuclei in the embryo to examine whether the yolk syncytial layer formed correctly after 2-OST knockdown with morpholinos. In both the control (Fig. 3.1A) and the 2-OST morphant embryos (Fig. 3.1B) it is clear that the YSL formed properly as indicated by the presence of nuclei in the YSL. In WT and control MO embryos, the YSN bundle up towards the margin at the initiation of epiboly, and then extend both vegetally (external or eYSN) and under the blastodisc (internal or iYSN) (Fig. 3.1A, E). However, the 2-OST morphants fail to exhibit this bundling towards the margin (Fig. 3.1B, F), the YSN never undergo epiboly but remain arrested near the margin. Next, anti-α-tubulin was used to examine the microtubule organization

Figure 3.1. 2-OST controls organization and bundling of microtubules in yolk syncytial layer. Embryos were injected at the 1-2 cell stage with either control or 2-OST MO and fixed at 4 hpf using microtubule stabilizing buffer. Sytox green stains the nuclei in control MO (A) and 2-OST MO (B) embryos. Antibody against α-tubulin stains microtubules in the blastodisc and yolk of control MO (C) and 2-OST MO (D) embryos. The red lines indicate the width of the YSL. Merged sytox green (red) and anti-α-tubulin (green) reveal narrow YSL in control morphant (E) embryos compared to wide YSL in 2-OST morphants (F). (n=25 embryos for each group) Scale bars are 100 um.

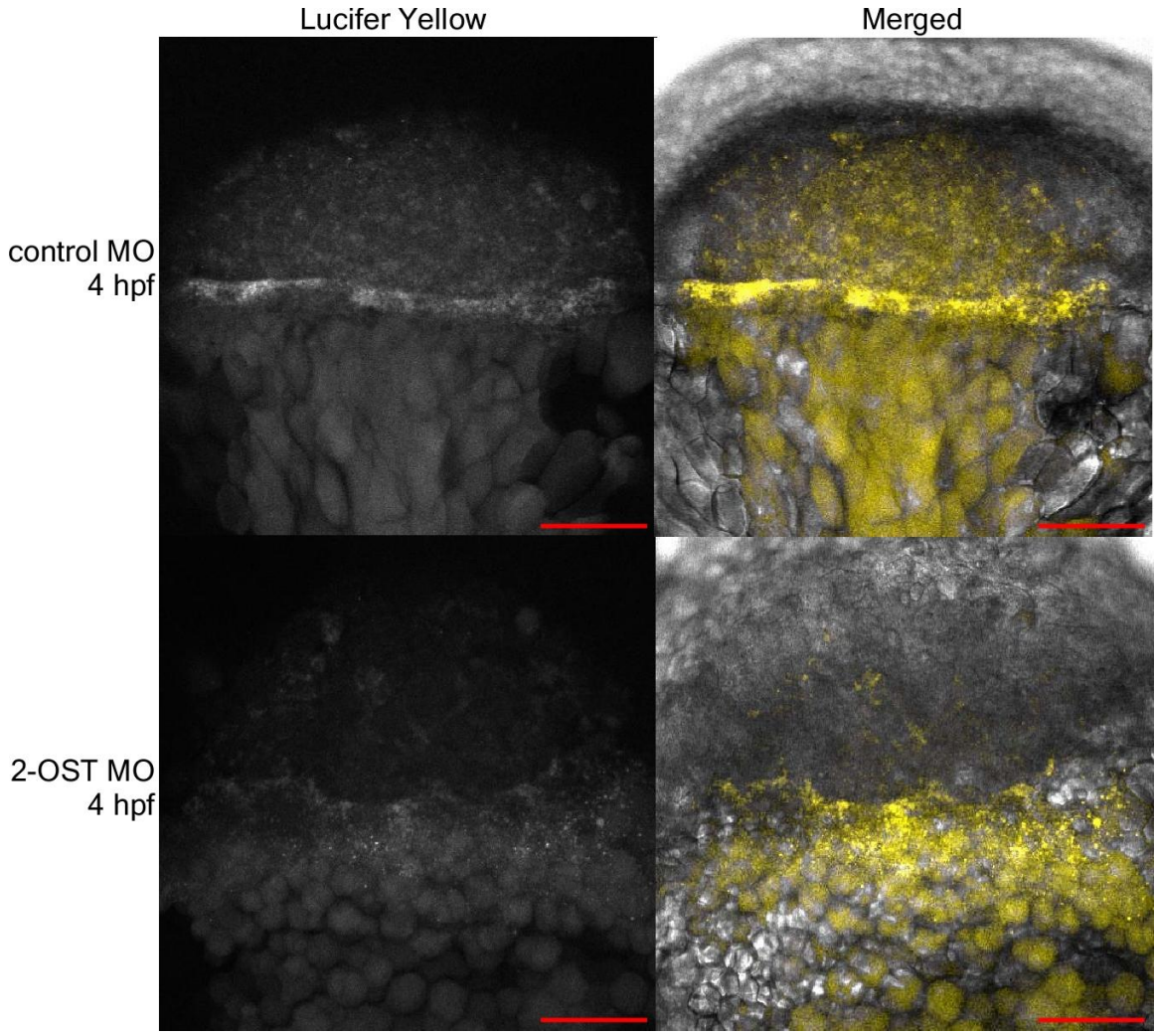

sytox green
anti-alpha-tubulin
merged
A
C
E
B
D
F
control MO
4 hpf
2-OST MO
4 hpf

in the yolk. In the WT embryo, the cortical array of microtubules which extends around the margin in the YSL was visible as were the microtubules radiating down towards the vegetal pole (Fig. 3.1C). By comparison, the 2-OST morphant microtubules were disorganized, clumped in some areas, and absent in others (Fig 3.1D). This suggests that at least part of the epiboly initiation defect is due to the disorganization of the microtubules in the YSN and furthermore that 2-OST is necessary for their correct organization.

2-OST Morphants Display Reduced Endocytosis

Another aspect of epiboly believed to be important for vegetal migration of the EVL is endocytosis of bits of the yolk membrane and with subsequent movement of these blebs of yolk further up into the animal pole of the yolk (Betchaku and Trinkaus, 1986; Solnica-Krezel and Driever, 1994). However, whether this endocytosis occurs to promote the doming or whether it is simply a secondary effect of the endocytosis is an area of contention in the field. Endocytosis of Lucifer Yellow in WT embryos was observed at the margin of the EVL and yolk (Fig 3.2A). However, the uptake of Lucifer yellow in 2-OST morphants occurred at a substantially lower rate (Fig. 3.2B). Previous studies have demonstrated that when microtubule organization is disrupted, endocytosis of the yolk membrane is impaired which may explain the endocytosis phenotype of the 2-OST morphant.

Figure 3.2. 2-OST morphants display reduced endocytosis. Embryos were injected at the 1-2 cell stage with either control or 2-OST MO then dechorionated and soaked in Lucifer Yellow solution. Lucifer Yellow uptake after 2 hours via endocytosis at the margin is visualized in control MO (A) and 2-OST MO (B) embryos. Merged Lucifer Yellow and bright field in reveal increased endocytosis in control morphant (C) embryos compared to 2-OST morphants (D). (n=9 embryos for each group) Scale bars are 100 um.

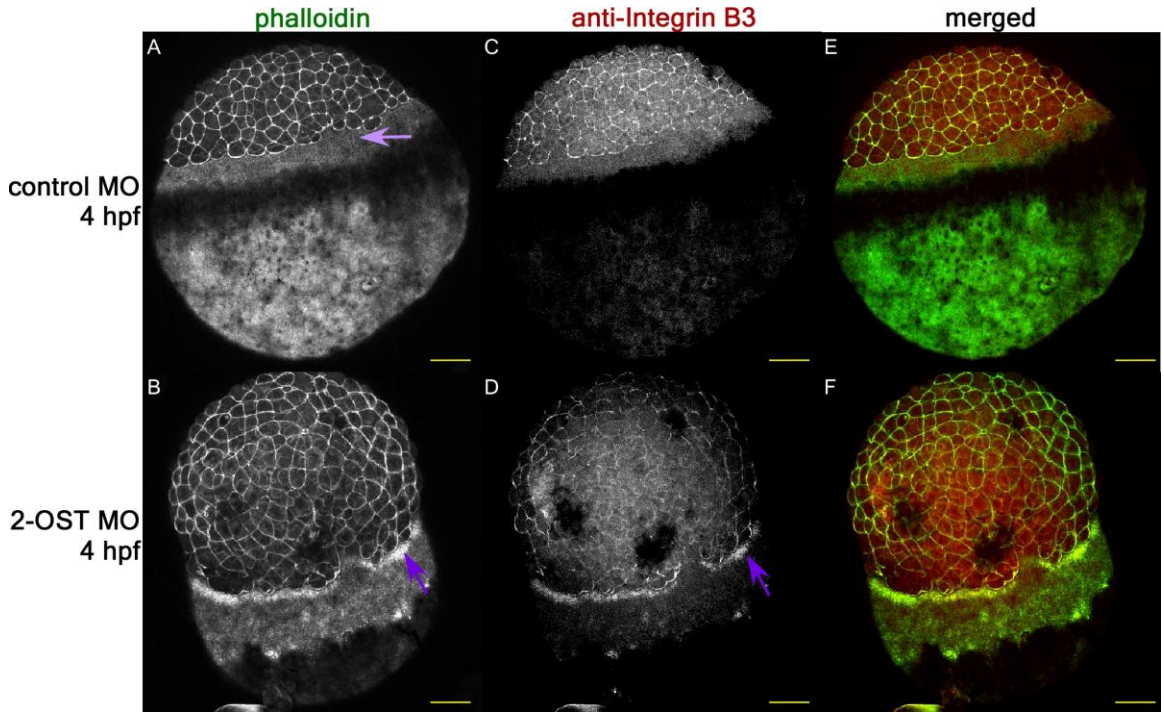

Lucifer Yellow
Merged
control MO
4 hpf
2-OST MO
4 hpf

2-OST Morphants Display Aberrant Actin Organization in Yolk Syncytial Layer

Actin also plays a key role in epiboly. Actin filamentation is important during the earlier stages of epiboly as it lends stability to the yolk cell and later in epiboly when it forms a drawstring at the margin, creating a tight band around the equator of the embryo that constricts to help draw the EVL down around the yolk towards the vegetal pole (Cheng et al., 2004; Koppen et al., 2006). This band does not normally form until after 50% epiboly, and plays an important role in epiboly progression. At dome stage, actin is normally organized into loose filaments at the margin and a mat at the vegetal pole (Fig. 3.3A), as previously reported (Cheng et al., 2004). However, in the 2-OST morphants at the sphere to dome stage transition, a large, aberrant band formed in the YSL (Fig. 3.3B) while the actin mat normally found at the vegetal pole was dramatically reduced. The amount of filamentous actin at the cell membranes in the blastodisc often appeared to be increased as well.

Integrins are transmembrane cellular receptors composed of dimerized α and β subunits that facilitate both signaling from the ECM to inside the cell as well as allowing the cell to communicate from the inside out (Hynes, 2002; Luo et al., 2007). They play an important role in connecting the ECM to the cytoskeleton, maintain close relationships with HSPGs in the ECM, and are likely to play a role in the cell movements of epiboly. Seven β subunits have been identified thus far in fish but few studies thus far have focused on the role of integrins in early development (Ablooglu et al., 2007). Integrin β3 most frequently functions as part of a fibronectin receptor, and while its levels in early

Figure 3.3. 2-OST morphants display aberrant actin organization in yolk syncytial layer. Phalloidin stains the filamentous actin in control MO (A) and 2-OST MO (B) embryos. Integrinβ3 was visualized in the blastodisc and yolk of control MO (C) and 2-OST MO (D) embryos using primary and secondary antibodies. The dark purple arrows point to the aberrant filamentous actin and co-localized Integrin β3. The light purple arrow indicates the normal level of filamentous actin in the YSL. Images of phalloidin (green) and Integrinβ3 (red) merged in control morphant (E) embryos and 2-OST morphants (F). (n=25 embryos for each group). Scale bars are 100 um.


phalloidin
anti-Integrin B3
merged
A
C
E
B
D
F
control MO
4 hpf
2-OST MO
4 hpf

development were quite low (Fig. 3.3C,E), in the 2-OST morphants Integrin β3 appeared to be upregulated, and was colocalized with the aberrant actin band (Fig. 3.3D,F).

2-OST is Necessary for Epiboly Progression

2-OST is necessary for the initiation of epiboly. To examine whether 2-OST is necessary for epiboly *progression*, 2-OST MO was injected into the yolk at the dome stage (4.5 hpf). During this stage the cytoplasmic bridges between the blastodisc and yolk no longer exist (Kimmel and Law, 1985b), thus restricting the knockdown of 2-OST exclusively to the yolk. These embryos arrested in epiboly, usually around 7-8 hpf, exhibiting a rounded EVL morphology at the margin, suggesting the cells are experiencing reduced circumferential tension.

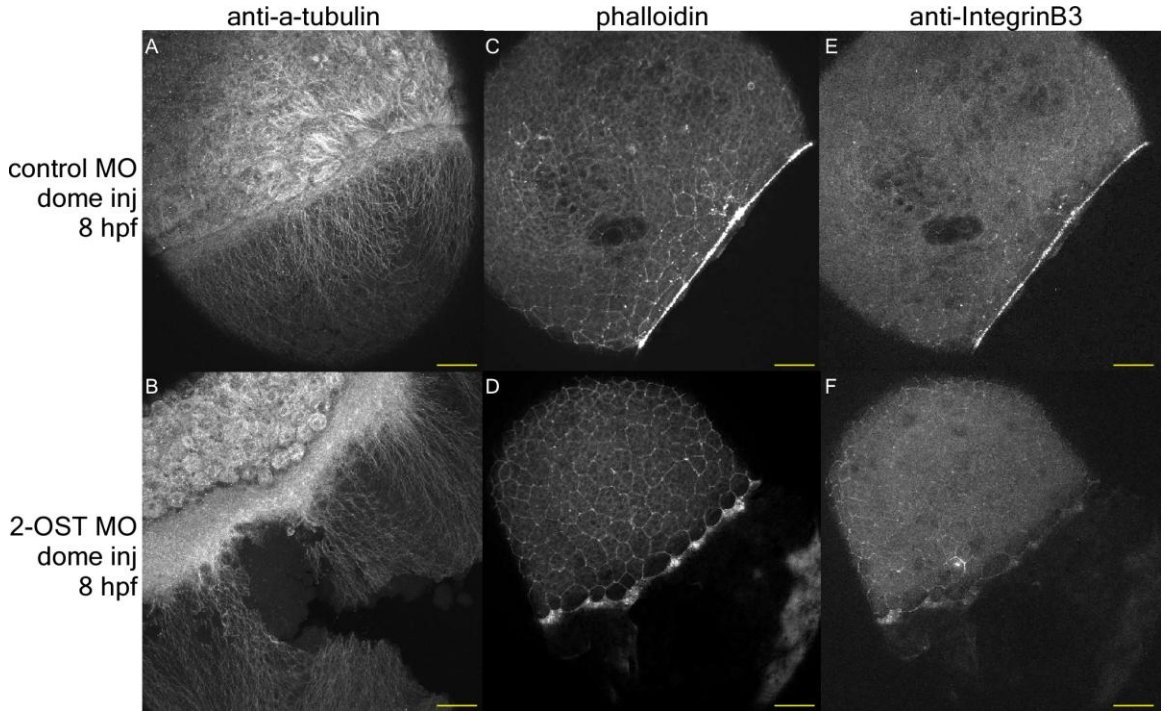
Initially we wanted to address if reduced microtubule function was contributing to the epiboly arrest and thus contributing to the loss of tension. Examination of the microtubules in the yolk of WT embryos at 8 hpf epiboly revealed a very narrow cortical band at the margin with microtubules radiating towards the vegetal pole (Fig.3.4A). By comparison the microtubules of the dome stage-injected 2-OST morphant embryos more closely resembled the whole embryo knockdown, with a wider cortical array and patchier vegetally radiating microtubules (Fig. 3.4B). This finding is consistent with studies using pharmacological and UV disruption of the yolk microtubules (Betchaku and Trinkaus, 1986; Strahle and Jesuthasan, 1993; Solnica-Krezel and Driever, 1994), and suggests that correct microtubule organization and function are essential not only for epiboly initiation but progression as well. Furthermore, our findings indicate that 2-OST plays an important role in regulating both epiboly and cytoskeletal structures.

We then wanted to see if the actin phenotype in the yolk knockdown was contributing to the epiboly arrest or if the drawstring formed properly. By 8 hpf the actin drawstring has formed in WT embryos (Fig. 3.4C). The co-localization of Integrin β3 with actin suggests this protein may play a role in the drawstring activity of the actin band (Fig. 3.4E). In contrast to the whole embryo 2-OST knockdown (Fig. 3.2B), the yolk knockdown does not appear to form the same aberrant actin band, although the drawstring also does not form properly. Instead there are patches of actin filamentation in the YSL which co-localize with Integrin β3 as well (Fig. 3.4F). This suggests that epiboly initiation and epiboly progression are regulated by some common as well as some different elements by 2-O-sulfation. Furthermore, it suggests that the blastodisc is not essential to the regulation of epiboly exercised by 2-O-sulfation.

2-OST Modulates FGF Signaling

To identify effects of 2-OST on individual players in major signaling networks we performed a microarray analysis comparing WT with 2-OST morphants. Previous studies demonstrate that interfering with FGF signaling at an early stage in development can block the initiation of epiboly (Krens et al., 2008b). Consequently, results revealing that a variety of components of the FGF signaling cascade were altered at the initiation of epiboly in 2-OST morphants was of particular interest. To validate these results we performed *in situ* hybridizations examining all aspects of the cascade. According to array data the Fgf17b ligand was down 6-fold (Fig. 3.5A,B), and FgfR2 was down 13-fold in morphants (Fig. 3.5C,D), suggesting reduced FGF signaling. Expression levels of a downstream target of FGF signaling, the transcription factor *pea3*, were down 4.7-fold in 2-OST morphants compared to WT (Fig. 3.5E,F). However, erm, a related transcription

Figure 3.4. 2-OST knockdown exclusively in yolk is sufficient to alter cytoskeletal elements. Embryos were injected at the dome stage with either control or 2-OST MO. Antibody against α-tubulin stains microtubules in control MO (A) and 2-OST MO (B) embryos. Phalloidin labels the filamentous actin in control MO (C) and 2-OST MO (D) embryos. Integrinβ3 was visualized in control (E) and 2-OST (F) morphant embryos using an antibody. (n=15 embryos for each group) Scale bars are 100 um.


anti-a-tubulin
phalloidin
anti-IntegrinB3
control MO
dome inj
8 hpf
2-OST MO
dome inj
8 hpf
A
B
C
D
E
F

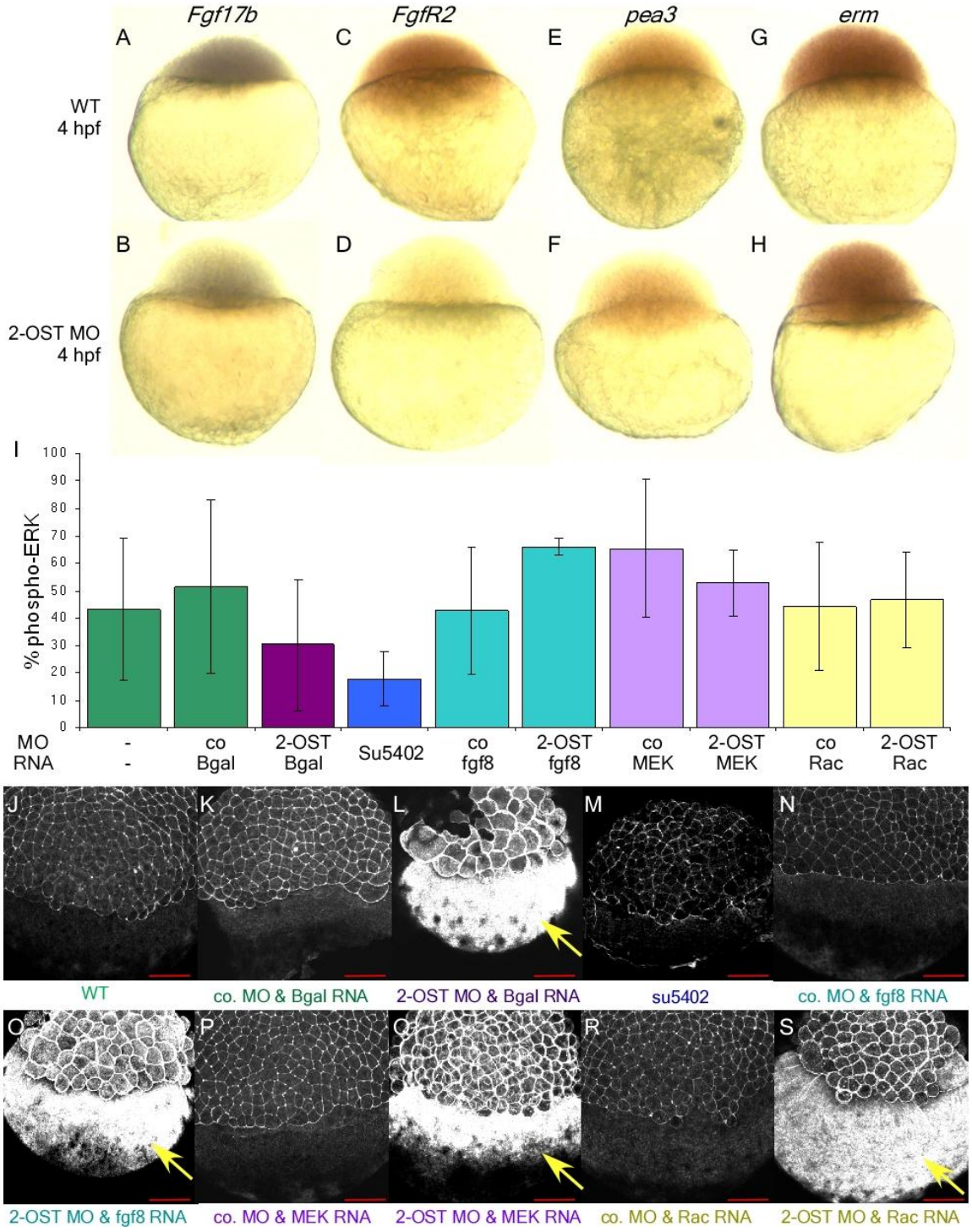
factor, had no significant different in expression levels between WT and 2-OST morphant (Fig. 3.5G,H).

Phosphorylated ERK is a well-known indicator of active FGF signaling (Krens et al., 2008b). Western blot analysis of whole embryos indicated that the levels of phosphorylated ERK relative to the total levels of ERK were reduced in 2-OST morphants compared to WT embryos and control morphants (Fig. 3.5I). The lowest levels of phospho-ERK were observed when embryos were treated with SU5402, an inhibitor of FGF signaling, at concentrations sufficient to block epiboly initiation.

Epistasis studies were carried out to determine where in the FGF signaling cascade 2-OST function is required. Ectopic expression of FGF8 demonstrated a strong rescue of ERK activation in the 2-OST morphants, as well as in the control morphant embryos (Fig. 3.5I). Expression of a constitutively active MEK (caMEK) also strongly rescued ERK signaling. Expression of a constitutively active Rac (caRac) also rescued ERK phosphorylation in the 2-OST morphants but at a more reduced level with the level of ERK activation similar to that of the WT and control morphants. This suggests that the absence of 2-OST increases permissiveness of FGF8 signaling to stimulate the intracellular FGF pathway compared to WT embryos levels of 2-O-sulfation, lending further support to the idea that changes to sulfation patterns not only block some signaling cascades but also promote others.

In spite of the rescue of ERK activation, none of these manipulations led to a rescue of the epiboly initiation defect. In a previous study where blocking FGF signaling *via* SU5402 led to an epiboly initiation defect, the authors found the microtubules were disorganized but did not look at the actin (Krens et al, 2008b). To try and elucidate the

Figure 3.5. Role for FGF signaling in altered cytoskeleton. *In situ* hybridization was performed on 4 hpf WT and 2-OST morphant embryos for the FGF ligand *fgf17b* (A,B), the Fgf receptor *fgfr2* (C,D), and the downstream FGF transcription factors *pea3* (E,F) and *erm* (G,H). Western blots were performed to examine epistatic FGF relationships in control MO and 2-OST MO embryos at the initiation of epiboly comparing levels of phosphorylated ERK relative to the total levels of ERK using the combinations of MO and RNA injected (I) (n=1 embryo/lane/sample, experiments were repeated in triplicate). WT and SU5402 treated embryos were used as controls. Phalloidin was used to examine the effect of ERK rescue upon the cytoskeleton of 2-OST morphants. WT (J), control MO injected with B-gal as a control (K), as well as control MO injected with fgf8 (N), consitutively active MEK (P), or consitutively active Rac (R) RNA all showed no difference between their levels of filamentous actin, as indicated by the yellow arrows. Embryos treated with SU5402 showed slightly reduced levels of actin by comparison (M). No difference was observed between 2-OST MO embryos injected with Bgal as a control (L), fgf8 (O), constitutively active MEK (Q), or constitutively active Rac (S) RNA. (n=9 embryos for each group) Scale bars are 100 um.


Fgf17b
FgfR2
pea3
erm
A
C
E
G
WT
4 hpf
B
D
F
H
2-OST MO
4 hpf
I
% phospho-ERK
100
90
80
70
60
50
40
30
20
10
0
MO
RNA
-
-
co
Bgal
2-OST
Bgal
Su5402
co
fgf8
2-OST
fgf8
co
MEK
2-OST
MEK
co
Rac
2-OST
Rac
J
K
L
M
N
WT
co. MO & Bgal RNA
2-OST MO & Bgal RNA
su5402
co. MO & fgf8 RNA
O
P
Q
R
S
2-OST MO & fgf8 RNA
co. MO & MEK RNA
2-OST MO & MEK RNA
co. MO & Rac RNA
2-OST MO & Rac RNA

origins of the aberrant filamentous actin in the yolk we compared WT (Fig. 3.5J), control MO (Fig. 3.5K) and 2-OST MO (Fig. 3.5L) embryos to SU5402 embryos (Fig. 3.5M) that exhibited an epiboly initiation defect and found that aberrant actin filaments were not observed when FGF signaling was reduced. Furthermore, none of the combinations that rescued the ERK-phosphorylation defects seen in 2-OST MO embryos [2-OST + fgf8 RNA (Fig. 3.5O); 2-OST + caMEK (Fig. 3.5Q) and 2-OST + caRac (Fig. 3.5S)], rescued the aberrant actin phenotype. It seems that although 2-O-sulfation may contribute to FGF signaling, it is not the immediate cause of the epiboly initiation defect.

In our previous study we looked at canonical Wnt activation and found that activation of that pathway also failed to rescue the aberrant actin band (data not shown). Therefore, the 2-OST morphant has a complex phenotype regulating many different cascades, some of which contribute to the epiboly initiation defect and the altered cytoskeletal components.

Syndecans are Required for Epiboly Initiation and Proper Organization of Actin

A wide variety of HSPGs are expressed maternally and via early zygotic transcription. Attempting to identify the core proteins that 2-OST might be mediating its effects, a variety of genes were knocked down and examined for epiboly defects. Simultaneous knockdown of Syndecans (Sdc) 2 and 4 (Fig. 3.6E) resulted in embryos which failed to initiate epiboly or arrested once epiboly had commenced and exhibited a loosely bundled YSN, bearing a strong resemblance to 2-OST MO embryos (Fig. 3.6B). By comparison, individual knockdown of either Sdc2 (Fig. 3.6C) or Sdc4 (Fig. 3.6D) failed to impede epiboly initiation and the YSN most strongly resembled the control MO

(Fig. 3.6A). Consistent with these observations, the Sdc2,4 double morphants (Fig. 3.6J) exhibited microtubule organizational defects that resembled the 2-OST morphants (Fig. 3.6G). The individual Sdc MO (Fig. 3.6H,I) displayed organized microtubules like those seen the in the control embryos (Fig. 3.6F).

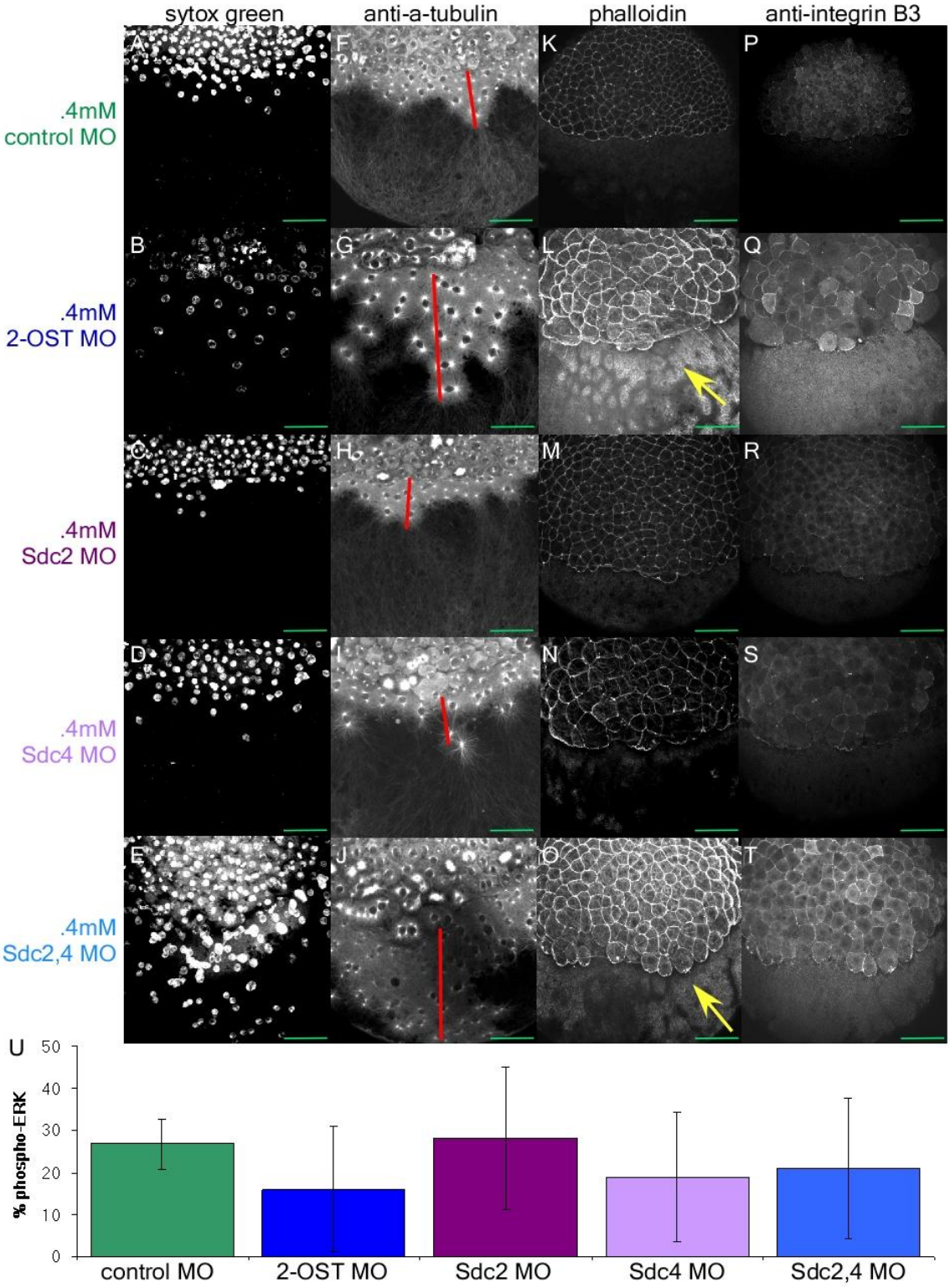
The double knockdown of the Sdcs also produced the aberrant filamentous actin phenotype (Fig. 3.6O) and co-localization of Integrinβ3 (Fig. 3.6T) which were seen in the 2-OST MO embryos (Fig. 3.6L,Q). Again, the single Sdc2 MO (Fig. 3.6N,R) and Sdc4 MO (Fig. 3.6N,S) knocked down did not display these aberrant cytoskeletal components and instead exhibited a phenotype more consistent with the control morphants (Fig. 3.6K,P). From these results it seems plausible the syndecans are one of the most significant targets of 2-O-sulfation in modulating the cytoskeletal rearrangements that contribute to the epiboly initiation defect.

To examine if syndecan deficiencies are modulating FGF signaling as well, the proportion of phopho-ERK activation relative to the total levels of ERK was measured via Western blot and compared to the control and 2-OST morphant embryos (Fig. 3.6U). ERK activation was reduced in both the Sdc4 morphants as well as in the Sdc2,4 double morphants. Taken together it appears that both the organization of the cytoskeletal constituents and the FGF signaling cascade are modulated in part by the syndecans.

Discussion

Our previous studies examined the contribution of Wnt signaling to cell adhesion and proliferation in the blastodisc when 2-O-sulfation is reduced (see Chapter 2). However, while manipulation of the intracellular Wnt signaling rescued the adhesion and proliferation defects, it did not rescue the epiboly initiation defect. In this study we

Figure 3.6. Syndecans are required for proper organization of actin. Epiboly initiation was tracked in Sdc morphants by examining bundling of the YSN by sytox green nuclei stain (A-E) and microtubule organization organization was tracked using anti-α-tubulin (F-J). The YSN fail to bundle up near the margin, indicated by the lengths of the red lines, and the microtubules are disorganized in the 2-OST MO (B,G) and Sdc2,4 MO (E,J) embryos, and both fail to initiate epiboly, compared to the control MO (A,F), Sdc2 MO (C,H), and Sdc4 MO (D,I) do (n=9). Furthermore, examination of the filamentous actin by phalloidin (K-O) and Integrinβ3 using an antibody (P-T) reveal that the aberrant actin band forms in 2-OST MO (L,Q) and Sdc2,4 MO (O,T), indicated by the yellow arrows, embryos which appears to co-localize with the Integrinβ3, while the control MO (K,P), Sdc2 MO (M,R), and Sdc4 MO (N,S) display normal actin organization and Integrinβ3 (n=9 embryos for each group). A chart compares the level of active, phosphorylated ERK to the total level of ERK, measured by Western blot and normalized to control levels of αtubulin at 4 hpf , for control MO, 2-OST MO, Sdc2 MO, Sdc4 MO, and Sdc2,4 MO (n=1 embryo/lane/sample, experiments were repeated in triplicate) (U). Scale bars are 100 um.


sytox green
anti-a-tubulin
phalloidin
anti-integrin B3
.4mM control MO
.4mM 2-OST MO
.4mM Sdc2 MO
.4mM Sdc4 MO
.4mM Sdc2,4 MO
A
F
K
P
B
G
L
Q
C
H
M
R
D
I
N
S
E
J
O
T
U
% phospho-ERK
50
40
30
20
10
0
control MO
2-OST MO
Sdc2 MO
Sdc4 MO
Sdc2,4 MO

sought to further characterize the epiboly initiation defect observed when 2-OST function was reduced and attempt to identify what other pathways might be contributing to this compound phenotype.

2-O-Sulfation Controls Cytoskeletal Organization in the Yolk

We first looked at the cytoskeletal components previously shown to play important roles in epiboly. Microtubules are necessary to draw the YSN and EVL vegetally in epiboly (Strahle and Jesuthasan, 1993; Solnica-Krezel and Driever, 1994). Proper actin organization is essential for both the stability of the yolk cell as well as the remodeling and constriction of the EVL during epiboly (Zalik et al., 1999; Cheng et al., 2004). Interfering with any of these processes, through physical or chemical manipulation, results in arrest of epiboly. In the 2-OST morphants the organization of both the microtubules and the actin is disrupted. Microtubules are present in the yolk of 2-OST morphants but fail to function properly, never undergoing the normal animal-vegetal axis narrowing that occurs at the initiation of epiboly. As a consequence, the YSN fail to migrate vegetally or under the blastodisc. The failure of the microtubules to function properly is consistent with the reduced rate of yolk membrane endocytosis also observed in the 2-OST morphants. Previous studies have demonstrated that when microtubule function is impaired, so is the endocytosis (Solnica-Krezel and Driever, 1994). Intriguingly however, microtubules do not appear to be disrupted in the cells of the blastodisc as networks of microtubules are visible throughout the cytoplasm of these cells. Additionally, as our previous studies show cell division occurs properly, albeit at a slower rate. This suggests perhaps that different pathways regulate microtubule stability in the yolk compared to the microtubules in the blastodisc.

Actin organization is also disrupted in 2-OST morphants, though in a manner previously undocumented in the epiboly literature. Instead of a thin band of filaments at the margin and a large mat of actin at the vegetal pole, the 2-OST morphants display an expanded domain of filamentous actin near the margin while the vegetal actin mat is dramatically reduced or nearly absent. We have observed that many of the 2-OST morphants die by lysis of the yolk cell between 4 hpf and 10 hpf, generally the window during which epiboly is occurring. This suggests that perhaps the actin band which forms near the margin possesses some contractile capacity and also that the yolk cell may be more prone to rupturing because cell stability is compromised due to the disorganized actin.

2-O-Sulfation is Necessary for Both Epiboly Initiation and Progression

While a number of genes have been shown to play a role in epiboly progression, only a handful of genes have been shown to play a role in initiating epiboly. Even fewer have been shown to play a role in both of these processes (Reim and Brand, 2006; Huang et al., 2007; Lachnit et al., 2008). Knockdown of 2-OST throughout the whole embryo results in a failure to initiate epiboly, while knocking down 2-OST only in the yolk results in an arrest of epiboly. This suggests that the pathways modulated by these changes to the HSPG GAG chains are important for regulating the organization and remodeling of the actin and microtubules in development.

In both whole embryo and yolk-specific knockdown of 2-OST, alterations in the levels of 2-O-sulfation result in changes to the cytoskeleton. The microtubules appear disorganized, though present and vegetal migration of the YSN fails to begin or arrests.

However, the actin organization appears to be different between the two experimental conditions. The large disorganized band of microfilaments which forms near the margin in the YSL of the whole embryo knockdown animals is not observed in the yolk-only knockdown. Instead, in the latter, the actin appears to frequently cluster at the regions where the EVL cells contact each other and the yolk but fails to organize into a drawstring.

Remodeling of the EVL has also been shown to play an important role in the progression of epiboly, requiring the recruitment of actin and myosin II to the EVL-yolk interface (Koppen et al., 2006). Failure to undergo correct remodeling may be contributing to the epiboly arrest phenotype observed in the yolk-restricted 2-OST knockdown. Furthermore, the cells of the EVL adjacent to the yolk fail to both flatten out and remodel forming a flush line at the margin, suggesting that epiboly fails in these morphant embryos in part because of a remodeling defect. This may also be due to a lack of tension upon the EVL via the yolk due to the loss of proper microtubule organization.

The different behavior of the actin in these two sets of morphants suggests that 2-OST whole embryo morphants may have an altered sense of timing. The aberrant band of actin which appears near the margin around 4 hpf may be an attempt to form the drawstring which normally is organized several hours later in development. Based on the immunohistochemistry results which suggest Integrinβ3 is localized to the drawstring, it is plausible that altering 2-O-sulfation patterns alters the pattern and timing of Integrinβ3 expression. This aberrant Integrinβ3 expression may then lead to increased actin filament organization. The only study to look at Integrinβ3 thus far in fish suggests by RT-PCR that this gene is not expressed until later in development than the stage we are looking at

(Ablooglu et al., 2007). However, fibronectin fibrillogenesis begins to occur roughly around the same time the drawstring is starting to form (Latimer and Jessen). As Integrinβ3 plays an important role coupling extracellular fibronectin signaling to the actin cytoskeleton, it seems likely that expression of Integrinβ3 occurs earlier than previously suggested. Further studies need to be performed to address this hypothesis. It also suggests that the organization of the microtubules and actin are not directly dependent upon one another because in the two morphants, the same outcome is observed for the microtubules while the organization of actin differs substantially. This is significant because in many developmental systems the organization and stability of these two cytoskeletal elements depend on one another.

2-OST Necessary for Proper FGF Signaling

HSPG and FGF interactions are one of the better characterized signal transduction pathway interactions in the current literature (McMahon et al.). HSPGs are thought to help facilitate the interaction between ligand and receptor, with changes to the GAG chains altering the affinity and subsequent success of these interactions (Johnson et al., 2006; O'Connell et al., 2007). Studies using engineered oligomerized sugars as well as *in vivo* models have shown that changing levels of 2-O-sulfation alters the affinity of different FGF ligands for their receptors (Ashikari-Hada et al., 2004; Kobayashi et al., 2007). In conjunction with this, previous studies knocking down ERK2 function and blocking FGF signaling *via* SU5402 have demonstrated a role for ERK and FGF signaling in epiboly initiation (Krens et al., 2008b).

Our microarray and *in situ* results comparing levels of RNA in WT and 2-OST morphants at the initiation of epiboly showed altered RNA levels of FGF ligands and

receptors and signaling co-factors. This suggests defects in the FGF signaling cascade might be contributing to the epiboly initiation defect. Demonstrating the complexity of this pathway, as well as the alterations in behavior elicited by changes in 2-O-sulfation, one measure of FGF signaling activation, the transcription factor *pea3*, was down in 2-OST morphants, while no significant change was seen in the levels another FGF transcription factor, *erm*. This suggests that while some components of the FGF pathway are down, others may not be. In zebrafish there are currently 29 FGF ligands, though not all of them are expressed in early development (Itoh, 2007). Given the complex nature of HSPG signaling and FGF signaling feedback loops, it is highly unlikely that modifications to 2-O-sulfation would affect all FGF ligands the same way.

Phosophorylated ERK is often used as a measure of FGF signaling activity at the protein level. We compared levels of phosphorylated ERK in WT, control, and 2-OST morphants and found 2-OST morphants had reduced levels of phospho-ERK while the total levels of ERK were fairly consistent in all three groups. While ERK activity can be regulated by FGF signaling, other pathways are also thought to converge upon this signaling molecule (Schier and Talbot, 2005; Krens et al., 2008a). This reduced level of ERK activation led us to examine the epistatic relationships upstream and downstream of the ligand-receptor interaction to see whether we could rescue the levels of activated ERK as well as the epiboly initiation defect.

Expression of a constitutively active MEK RNA, an intracellular component of the FGF signaling network, increases levels of phospho-ERK in both the control and 2-OST morphants. MEK directly phosphorylates ERK so these results are consistent with the expected outcome. Expression of constitutively active Rac, a GTPase which interacts

with a variety of signal transduction pathways including FGF, has no effect on the phospho-ERK levels in control morphants, but rescues the 2-OST morphant phospho-ERK levels. ERK is just one of the many targets of Rac activity, and Rac is activated by a variety of pathways, but it suggests that the effects of 2-O-sulfation are modulated upstream of Rac activity. Ectopic expression of FGF8, a secreted extracellular FGF ligand, with the control morpholino did not produce a consistent change in levels of ERK activation. However, fgf8 RNA over-expression in 2-OST morphants led to a dramatic increase in ERK activation. This suggests that the absence of 2-OST increases affinity of the ligand-receptor interaction, leading to increased ERK-phosphorylation. This seems counter-intuitive at first. However, FGF8 is expressed in a limited number of cells at the initiation of epiboly (Maegawa et al., 2006), and in these experiments translation of FGF8 is occurring throughout the whole embryo, so it is not representative of what is occurring in a normal embryo or 2-OST morphant embryo normally at that time. Therefore, even though this may not be representative of normal development, it does serve its function in the epistasis study to try to identify where determine where in the FGF signaling cascade 2-OST function is required. These results show that 2-O-sulfation is important at the level of ligand-receptor interactions and that 2-O-sulfation is important to FGF signaling.

Surprisingly, although ERK activation levels were rescued with a variety of manipulations, none of them rescued the epiboly initiation defect. This suggests that reduced ERK activity alone is not causing the 2-OST MO epiboly defect. As was stated earlier, many different families of genes support epiboly and so while ERK contributes to epiboly initiation, in the 2-OST morphant it is not the sole source of the epiboly initiation defect. Furthermore, none of the ERK manipulations had any effect on the aberrant actin

band in the YSL, confirming that reduced ERK activity is not directly responsible for that alteration in the cytoskeleton. It is worth noting that hyper-activated Rac activity is thought to speed up rates of epiboly (Leskow et al., 2006) and is known to modulate cytoskeletal rearrangement (Hall, 1998). However, that perhaps indicates that Rac has an important role in epiboly progression, but not necessarily epiboly initiation.

Phospho-ERK is localized to the marginal EVL and deep cells during epiboly initiation. Blocking ERK2 blocks the initiation of epiboly (Krens et al., 2008b), suggesting a dialogue between the cells of the blastodisc and the yolk contributes to this initiation in part by orienting, organizing or stabilizing microtubules. However, as rescuing total phoshpo-ERK fails to rescue epiboly in our morphants, we speculate that the localization of phospho-ERK was global but still not sufficient in the necessary cells. Alternatively, the epiboly defect arises from a compound phenotype, and thus other molecules and pathways are involved. Furthermore, as rescuing ERK function *via* several different methods all fail to impact the aberrant actin organization in the yolk, we speculate that other pathways are involved in the organization of yolk filamentous actin.

Syndecans Are Necessary for Correct Actin Organization in the Yolk

To try to identify which HSPG core proteins might be the target of 2-OST activity and thereby contribute to the epiboly initiation defect, we began experimenting with different combinations of core protein morpholinos. Syndecans are a family of single-pass transmembrane core proteins. This family is characterized by GAG chains in the extracellular matrix most frequently decorated with HS disaccharides and modifications, a transmembrane domain capable of dimerization, and a cytoplasmic tail with an NXIP

motif which promotes integrin interactions and a PDZ domain that interacts with a wide range of proteins (Bass and Humphries, 2002; Bass et al., 2009). Exciting work has been done in cell culture and other *in vivo* systems to try to understand the interaction between syndecans and integrins which cooperate to regulate both ECM and cytoskeletal organization (Bass and Humphries, 2002; Xian et al.). We found that knocking down Sdc2 and Sdc4 individually failed to produce an epiboly initiation defect, but knocking them down synchronously resulted in a failure to initiate epiboly, disorganized microtubules and unbundled YSN in the YSL, and most significantly, increased filamentous actin localized to the margin. These results suggest that syndecans play a crucial role in the organization of actin in early zebrafish development. They also appear to facilitate FGF signaling based on phospho-ERK levels.

Syndecans are thought to homodimerize as well as heterodimerize (Choi et al., 2005), interactions which might be promoted or disrupted based on GAG chain modifications and thus resemble the 2-OST phenotype when they are knocked down together by morpholino. Alternatively, their ability to interact as they would under normal developmental conditions with other proteins may be altered by these GAG chain modifications, resulting in the alterations seen in the cytoskeleton. Further studies are necessary to understand how these core proteins are facilitating these interactions.

In this study we have demonstrated a novel role for 2-O-sulfation in the regulation of cytoskeletal elements which control epiboly initiation and progression. We have begun to identify the targets of these 2-OST modifications which contribute to this defect and find they include Syndecans 2 and 4. We also found that 2-OST plays a role in regulating FGF signaling in early development but that reduced FGF signaling does not

appear to directly contribute to the epiboly initiation defect. These results continue to build the story of the complex phenotype due to 2-OST knockdown. Future studies will address other pathways potentially affected by 2-O-sulfation pattern changes.

Materials and Methods

Zebrafish Lines

Oregon AB wild-type zebrafish (Danio rerio) were maintained on a 14-hour light/10-hour dark cycle at 28.5 degrees Celsius. Zebrafish embryos resulted from natural spawning and were collected, injected, raised, and staged as previously described (Essner et al., 2005).

Morpholino and RNA Injection of Zebrafish Embryos

The control and 2-OST MO have been previously published (Cadwalader et al., in prep). Embryos were injected at the 1-2 cell stage. Sydencan-2 MO was previously published by Cam Arrington and the Sydencan-4 MO and plasmid were gifts from Ken Kramer. B-galactosidase mRNA was injected as a control (GenBank Accession number AP_000996). Ectopic expression of fgf constituents came from pCS2-fgf8, constitutively active Rac was a gift from Jeroen Bakkers and Matthias Hammerschmidt (Bakkers et al., 2004), constitutively active MEK was a gift from Malcolm Whitman (LaBonne et al., 1995). RNA was made using respective Message Machine kits (Ambion).

In Situ Hybridization

For *in situ* hybridization analysis embryos were fixed overnight at 4 degrees in sucrose buffered 4% Paraformaldehyde. They were subsequently rinsed in phosphate buffered saline (PBS), dehydrated in methanol, and stored at -20 degrees. Clones

encoding the open reading frame (ORF) of *fgf17b*, *fgfR2*, *pea3*, and *erm* were used to make probe via in vitro transcription. Antisense RNA was made from those linearized plasmids using Digoxigenin (DIG) RNA Labeling Kit (Roche, IN). *In situ* hybridizations were carried out according to a previously established protocol (Thisse et al., 1993) using a Biolane HTI *in situ* machine (Huller and Huttner AG, Tübingen, Germany). Embryos were cleared in 70% glycerol in PBST and photographed using with a Nikon SMZ1000 on a Leica MZ12 dissecting microscope. Digital images were processed using Adobe Photoshop and ACD systems Canvas.

Immunohistochemistry

Immunohistochemical analysis for actin was performed on embryos fixed overnight at room temperature in 4% formaldehyde. They were subsequently rinsed in PBST, dechorionated, and blocked for 1 hour in blocking solution (1% DMSO, 5% goat serum, .5% Triton-X in PBS). They were blocked overnight in parallel using aliquots from an Alexa 488-conjugated phalloidin and anti-mouse anti-Integrin β3 (1:100) in block solution. They were then washed with block solution and subsequently incubated overnight in secondary antibody, Alexa 647-conjugated goat anti-mouse (1:200, Invitrogen). The following day they were washed again and mounted in Slow Fade on cover slips and visualized using Olympus FV1000 XY. Images were analyzed using ImageJ software. Microtubule IHC was performed using the MSB (Schroeder and Gard, 1992), anti-mouse α-tubulin (1:1000, abcam), Alexa 647-conjugated goat anti-mouse, and sytox green nuclei stain (Invitrogen).

Western Blot

Injected embryos were collected in buffer containing Western blots were performed as previously described . Primary antibodies used were mouse anti-alpha-tubulin (AbCam, 1:7500) as a control, rabbit anti-ERK2 (SantaCruz, 1:1000), and mouse anti-phospho Erk (BD Bioscience, 1:500). Secondary antibodies used were HRP-conjugated goat anti-mouse and HRP-conjugated goat anti-rabbit (Jackson ImmunoResearch, 1:2000). Membranes were exposed using ECL+ Western Blotting Detection System from Amersham. Densitometry analysis was performed using AlphaEase FC.

Microarray

WT and embryos injected with 2-OST MO at 1-2 cell stage were collected at two stages, the first at 4 hpf as epiboly was beginning and the second group at 8 hpf. The RNA was isolated and purified. Analysis of this RNA was performed at the Microarray Core using Agilent Technologies microarray platform for zebrafish using an Agilent Bioanalyzer. Results of microarray analysis were analyzed using GeneSifter software (Geospiza, Inc.).

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CHAPTER 4

CONCLUSION

In this study we gained an insight into the role 2-O-sulfation plays in early development. 2-O-sulfation is necessary for the initiation and progression of epiboly. It contributes to the modulation of Wnt and FGF signaling as well as the proper organization and function of cytoskeletal elements. Syndecans 2 and 4 appear to be important targets of 2-O-sulfation which suggests an important role for HSPGs in cellular communication during early development.

HSPGs Modulate Cellular Communication

The process by which an embryo forms from a single cell into a whole organism is a complex process requiring many different cell behaviors including division, migration, and differentiation (Solnica-Krezel, 2006; Rohde and Heisenberg, 2007). These changes in cell fate and location depend upon cell communication. A cell must direct its own activities and also communicate with surrounding cells. Heparan Sulfate Proteoglycans (HSPGs) are a family of molecules found in the extracellular matrix (ECM) of all metazoan cells that help facilitate this communication (Esko and Selleck, 2002; Hacker et al., 2005). HSPGs can be attached to the surface or secreted into the ECM (Kramer and Yost, 2003). Proteoglycans are characterized by a protein core with sites for glycosaminoglycan (GAG) attachments, consisting of disaccharide repeats of

glucuronic acid and N-acetyl glucosamine. These GAG chains are modified with a variety of charged groups including sulfate and acetyl moieties which are added in a non-template driven fashion by multiple enzymes. This creates an astounding degree of complexity on one single GAG chain, let alone the complexity of all the HSPGs expressed on the surface of one cell (Lamanna et al., 2007). These specific modifications are believed to promote and impede different signaling interactions between ligands and receptors, establishing gradients for molecules and contributing to the overall structure of the ECM (Johnson et al., 2006; O'Connell et al., 2007; Fujita et al., 2010).

Altering the structure of the GAG chains by modulating the bioavailablity of the enzymes which modify them has been shown to have a range of effects on development (Ringvall et al., 2000; Merry and Wilson, 2002; Reijmers et al., 2010). The studies presented here have focused on one particular enzyme, namely the 2-O-sulfotransferase (2-OST) which catalyzes the transfer of a sulfate group from a 3'-Phosphoadenosine-5'-phosphosulfate (PAPS) donor to the second carbon position on the uronic acid. Knocking down 2-OST via morpholino in zebrafish embryos results in altered cell communication in multiple signaling cascades. This affects a variety of cell behaviors including cytoskeletal organization, adhesion, migration, and proliferation.

2-OST Controls Epiboly Initiaiton

Epiboly is the process by which a population of cells atop the yolk migrate towards the vegetal pole to envelop the yolk cell (Warga and Kimmel, 1990). Largely, the genes that control this process regulate components of the cytoskeleton. Microtubules in the yolk cell attach the most superficial layer of blastodisc cells, the enveloping layer (EVL), to the yolk and the vegetal pole. Shortening of the microtubules

controlled at the vegetal pole helps pull the cells of the blastodisc down around the yolk (Solnica-Krezel and Driever, 1994). Knocking down 2-OST in the developing zebrafish embryo results in a failure to initiate epiboly. In the 2-OST morphant embryos these microtubules are present in the yolk but are disordered and fail to function properly. Further examination revealed that the yolk syncytial nuclei (YSN) fail to migrate towards the vegetal pole, indicating that part of this epiboly initiation defect is due to a failure of the microtubules to shorten in the yolk.

Endocytosis of the yolk membrane is another mechanism important for epiboly progression (Betchaku and Trinkaus, 1986; Solnica-Krezel and Driever, 1994). The endocytosed blebs of membrane are thought to reduce the distance the migrating blastodisc has to cover en route towards the vegetal pole, as well as being trafficked towards the animal pole to promote rearrangement of the yolk in a way that supports the upward doming. This process largely fails to occur in the 2-OST morphants. Microtubules which are not properly organized in 2-OST morphants play an essential role in endocytosis and therefore the failure of this process is not surprising.

Another key element for epiboly progression is the correct organization of actin in the yolk. At the beginning of epiboly a thin region of filamentous actin is found near the margin of the EVL and yolk in the yolk syncytial layer (YSL), and a large mat of actin covers the vegetal pole (Zalik et al., 1999). After the advancing margin of cells reaches the equator of the yolk, a drawstring of actin forms, remodeling the cells at the leading edge and forming a ring that constricts to help draw the cells down towards the vegetal pole (Cheng et al., 2004; Koppen et al., 2006). In 2-OST morphants, a large network of filamentous actin is present in a band around the yolk margin, while the vegetal mat of

actin is largely absent. In spite of manipulating different pathways, including the canonical Wnt and FGF pathways, we have been unable to identify a direct interaction facilitated by 2-O-sulfation which contributes to this altered filamentous actin.

One protein which is possibly bridging the modified 2-O-sulfation patterns and aberrant filamentous actin is Integrinβ3. This is based on immunohistochemical results that suggest Integrinβ3 plays a role in the normal drawstring function in epiboly. Integrins are receptors that function to promote communication across the cell membrane, from the outside-in and the inside-out, as well as helping promote the organization and the growth of actin filaments (Hynes, 2002; Delon and Brown, 2007). Therefore modulating 2-OST activity may alter interactions essential for the timing of events in epiboly or the localization of elements important for this process. In the 2-OST morphants integrins may organize prematurely in the YSL while lacking other elements that are necessary for the total organization of the drawstring, therefore promoting the reorganization of populations of actin in the yolk from the vegetal mat to the YSL. This results in the aberrant filamentous actin band in the YSL and decreases the stability of the rest of the yolk because the actin in the vegetal mat is reduced.

Two methods of manipulation can be used to explore this hypothesis about altered, increased integrin activity, including dominant negative integrins and integrin inhibitors. Either of these approaches could help us answer questions both about whether Integrinβ3 plays an essential role in epiboly as well as its regulation in 2-OST morphants. Currently we only know the actin and Integrinβ3 co-localize but have not demonstrated that the increased filamentous actin is due to the presence of Integrinβ3. If interfering with the activity of the integrin complexes prevents the formation of the filamentous actin

band in the 2-OST morphants, then it suggests that altered 2-OST activity contributes to altering the expression of Integrinβ3. Our previous attempts to identify whether the microtubule or actin defect appears first suggest they occur right around the same window of time, but we have been unable to determine if the disruption of one alters the other. If manipulating the integrins successfully disrupts the actin band, this will help address the question of whether the microtubule disorganization is due to the altered actin expression. Therefore, if manipulating the integrin interactions rescues the microtubule organization in the 2-OST morphants, this suggests the altered actin organization disrupts the microtubules. However, if there is no difference in the microtubules upon manipulation of integrins in the 2-OST morphants, then it suggests those defects are independent and that the microtubule defect is caused by a different set of interactions due to 2-OST activity. Both of these outcomes would be interesting because it remains unclear how microtubules and actin interact to stabilize each other in this environmental context as well as others.

2-OST Controls Epiboly Initiation and Progression

While is it clear that 2-O-sulfation contributes to epiboly initiation, we wanted to see if it also regulates the progression of epiboly. The junctions between the yolk and blastodisc close before the initiation of epiboly, therefore injecting morpholino into the yolk at the start of epiboly restricts the knockdown exclusively to that region (Kimmel and Law, 1985). Using this technique would not only determine whether 2-OST was necessary for the progression of epiboly but also to help answer the question of whether the epiboly initiation defect was solely due to factors in the blastodisc, elements in the yolk, or both. Embryos injected with 2-OST MO into the yolk lead to epiboly arrest and

eventual death by lysis of the yolk cell, suggesting that 2-OST is important not only for epiboly initiation but also for progression of epiboly.

Examination of the cytoskeleton reveals that, similar to the whole embryo knockdown, when 2-OST MO is injected in the yolk the microtubules become disorganized and fail to function properly. Unlike in single cell injections the actin does not form a large band near the margin, but instead largely clusters at the junctions between the EVL cells and the yolk. These results suggest several important things. First, this shows that regulation of epiboly progression can be independent of the blastodisc. Secondly, it demonstrates that the mechanisms by which 2-OST controls epiboly initiation are not entirely identical to the mechanisms by which it regulates epiboly progression. This supports the current notion that regulation of epiboly initiation and progression share some but not all of the elements (Solnica-Krezel and Driever, 1994; Reim and Brand, 2006; Huang et al., 2007; Lachnit et al., 2008). Lastly, the filamentous actin band which forms in the whole embryo 2-OST morphant may be due to an altered developmental timing. Alternatively, the mechanisms which regulate this process may be due to some altered signals between the blastodisc and the yolk. As the effects of 2-OST knockdown are hypothesized to alter interactions on the cell surface, we sought to identify the signaling molecules involved by examining different signaling cascades.

2-OST Controls Wnt Signaling

Canonical Wnt signaling contributes to a variety of important cell behaviors during development including adhesion and proliferation (McEwen et al., 2000; Schier and Talbot, 2005; Rohde and Heisenberg, 2007). Knocking down 2-OST resulted in both

a reduction of total protein and reduced localization of β-catenin and E-cadherin, components of adherens junctions, to the cell membranes. Particularly evident was the loss of protein between the deep cells of the blastodisc and resulted in reduced adhesion between these cells. Co-injection of stabilized β-catenin RNA resulted in a rescue of the localization of both E-cadherin and β-catenin to the cell membrane. This subsequently rescued the adhesion defect between the individual deep cells as well as between the deep cells and the EVL. These results suggest that 2-OST modifications contribute to the stability of adherens junctions in the developing embryos.

2-OST knockdown also resulted in cell cycle defects whereby cell division proceeded at a slower rate in morphants as indicated by a reduced rate of pulse labeling BrdU incorporation. Co-injection of the stabilized β-catenin rescued the cell cycle defects in the 2-OST morphants. These results suggest that loss of β-catenin stabilization due to reduced 2-O-sulfation contribute to cell cycle control in addition to the adhesion defect. However, rescuing both proliferation and adhesion was not sufficient to rescue the epiboly initiation defect.

To provide a mechanism for the rescue of the 2-OST morphant phenotype by stabilized β-catenin we conducted epistasis experiments to determine where in the Wnt signaling cascade 2-OST function is required. We found that ectopic expression of Wnt8 RNA increased levels of β-catenin and E-cadherin in control morphants but failed to increase levels of β-catenin in 2-OST morphants. Injecting Wnt8 RNA also failed to rescue the cell cycle defects based on rates of BrdU incorporation in 2-OST morphants. However, expression of a dominant negative glycogen synthase kinase 3 (dnGSK3), which interferes with the β-catenin destruction complex and thus promotes β-catenin

stabilization, was sufficient to increase β-catenin and E-cadherin levels in the 2-OST morphant. In addition, it also rescued the cell cycle defects. This suggests that the effect of 2-OST activity occurs downstream of canonical Wnt ligand signaling but upstream of the intracellular components of the Wnt cascade. These results are consistent with modulation of communication across the cell membrane where the GAG chains are localized.

E-cadherin plays an important role in epiboly. Rescue of 2-OST morphants with stabilized β-catenin restored the E-cadherin protein levels and membrane localization. However, it failed to rescue the epiboly initiation defects. The β-catenin rescued 2-OST morphants failed to exhibit the behaviors such as radial intercalation, which would be expected by a rescue of E-cadherin function (Babb and Marrs, 2004; Kane et al., 2005). We hypothesized that the intercalation defects would be rescued even if the other components which regulate epiboly were not. Calcium signaling is important for the homotypic adhesion between E-cadherin molecules (Shapiro et al., 1995). This suggests that while E-cadherin may have localized to the cell membrane, it was perhaps unable to function properly due to reduced levels of extracellular calcium ions necessary to promote homotypic adhesion interactions. Therefore E-cadherin, while present and correctly localized, may not be functioning correctly due to some other part of the compound phenotype.

The different manipulations of the canonical Wnt pathway also had no apparent effect on the cytoskeleton. This result was surprising because microtubules localize to adherens junctions. One theory as to why epiboly was failing to initiate was that the reduced adherens junctions were failing to stabilize the microtubules (Perez-Moreno et

al., 2003; Shaw et al., 2007). However, as the rescue of the adherens junctions by co-injection of β-catenin failed to rescue the microtubule defect in the 2-OST morphants, it seems the microtubule organization defect is independent of the reduced adherens junctions. Another possibility is that if E-cadherin localized correctly but was not actively functioning, then perhaps the microtubules failed to organize correctly at those junctions. Furthermore, rescue of the levels of β-catenin had no effect on the filamentous actin band which forms in the yolk in the 2-OST morphants, suggesting that the loss of Wnt activity due to reduced levels of 2-O-sulfation is not directly responsible for the filamentous actin. Therefore, this suggests the epiboly initiation defect is due to a compound phenotype, and examination of other signaling pathways will be necessary to understand the compound effect of 2-OST knockdown.

2-OST Modulates FGF Signaling

Previous studies have demonstrated a role for components downstream of FGF signaling in epiboly initiation (Krens et al., 2008). Preliminary studies suggested 2-OST knockdown altered transcriptional regulation of members of the FGF family. Examination of levels of phospho-ERK, a downstream kinase indicative of active FGF signaling, revealed that levels of phospho-ERK were down in 2-OST morphants during epiboly initiation. Epistasis studies were carried out to determine where in the FGF signaling cascade 2-OST function is required and to see if the epiboly initiation defect could be rescued. Ectopic expression of FGF8 failed to increase phospho-ERK levels in the control morphant but did increase them in the 2-OST morphants. This suggests that the absence of 2-O-sulfation increased the interaction of this particular FGF ligand and its receptor to promote FGF signaling. By comparison, expression of a constitutively active

MEK (caMEK), a kinase which phosphorylates ERK, increased levels of phospho-ERK in both control and 2-OST morphants (LaBonne et al., 1995). Expression of a constitutively active Rac (caRac) rescued levels of phospho-ERK in the 2-OST morphants to WT levels but did not increase phospho-ERK in the control morphants (Bakkers et al., 2004). These results suggest that 2-OST activity is important at the level of the ligand-receptor interaction and that loss of 2-OST increases FGF signaling via FGF8.

Initially the FGF8 ligand results are difficult to interpret because it could be argued that ERK activation should increase in the 2-OST morphants if 2-OST levels did indeed increase permissiveness of FGF signaling. FGF8 is endogenously expressed in a small population of dorsal cells at the initiation of epiboly (Maegawa et al., 2006). Therefore, ectopic expression of FGF8 in all cells of the blastodisc in the WT and 2-OST morphants is not entirely a true representative of development at that stage. In these studies we are exploiting the *in vivo* developmental model to answer the question of where in the signaling cascade 2-OST activity is important. Therefore, these results suggest that the normal 2-OST modifications present in WT GAG chains may reduce the interactions between the FGF8 ligand and FGF receptor. However, when these GAG chains have less 2-O-sulfation, ectopic FGF8 is able to interact more easily with the receptor, resulting in increased ERK activation. These results support the hypothesis that 2-OST activity is important at the level of ligand signaling. There are approximately 30 FGF ligands in zebrafish and given the complex nature of HSPG modifications and interactions, it would not be reasonable to expect all FGF ligands to be impacted by the loss of 2-OST in the same manner (Itoh, 2007). Of the 30 FGF's only a few appear to be

broadly expressed in early development, including the FGF1, 2, and 17. Interactions with their receptors may be different in the presence of 2-OST than that of FGF8 which would explain why ERK activity is rescued in 2-OST morphants.

Examination of the effects of reduced FGF signaling on actin using an FGF inhibitor, SU5402 (Mohammadi et al., 1997), revealed that the total level of filamentous actin was lower when FGF signaling is inhibited than in WT. Expression of FGF8, caMEK, and caRac RNA rescued the levels of phospho-ERK in the 2-OST morphants but failed to rescue epiboly and also did not impact the aberrant actin band. It seems that 2-OST does modulate FGF signaling, but FGF signaling is not individually responsible for either the epiboly initiation defect or the increase in filamentous actin. As FGFs are known to interact with integrins (Harmer, 2006; McMahon et al.), it will be interesting to look at whether treating 2-OST morphants with SU5402 reduces the band of filamentous actin as well as the localization of Integrinβ3 in future studies.

It is also possible that cross-talk between the FGF and Wnt pathways is contributing to the reduced adhesion and reduced ERK activity (Huelsken and Birchmeier, 2001; Kawakami et al., 2001; Katoh and Katoh, 2006). Many elements are shared by both these pathways and if manipulations higher up in these signaling cascades can rescue protein levels in the pathways, there is a possibility of cross-pathway rescue, particularly at the GSK3 junction. It would be interesting to look at whether manipulating β-catenin increases levels of phospho-ERK in the 2-OST morphants and alternatively if increased phospho-ERK raises levels of β-catenin in the 2-OST morphants. This would not only imply a role for 2-OST activity in modulating cellular activity in this environmental

context, but would also inform how these pathways function normally in early zebrafish development.

Syndecans Are Necessary for Epiboly Initiation and Actin Organization

Altering the cell behavior through HSPG proteins can take on two forms by modifying the GAG chain motif or the composition of a cell's HSPG core proteins. To identify which core proteins might be most important in modulating the activity of 2-OST we knocked down different core proteins individually and in tandem. Individual knockdown of Syndecan-2 (Sdc2) and Syndecan-4 (Sdc4) by morpholino did not result in epiboly initiation defects. However, knocking them down simultaneously resulted in epiboly arrest and epiboly initiation defects. These initiation and arrest defects appear to be due to the cytoskeletal arrangement defects similar to those observed in the 2-OST morphants. The microtubules appear disorganized and fail to migrate towards the vegetal pole in the Sdc2,4 morphants. Additionally, the aberrant actin band at the margin was observed. This suggests that syndecans play an important role in regulating actin organization. As Integrinβ3 also co-localized with the actin in Sdc2,4 double morphants, it suggests that the same type of timing defect observed in the 2-OST morphants is occurring in the Sdc2,4 double morphants.

Interactions of integrins with syndecans have been well documented (Rusnati et al., 1997; McMahon et al.). These results suggest that syndecan interactions with integrins are necessary for the proper organization of actin and in the absence of syndecans, the integrins are incorrectly localized. An alternative hypothesis to the timing defect (the idea that the actin drawstring band is forming prematurely in the 2-OST

morphants) involves the localization of these molecules in normal development. Our hypothesis is that integrin localization to the actin mat is normally modulated by syndecans, a target of 2-OST activity. In the absence of those syndecans, the integrins aberrantly localize to the margin. To examine this hypothesis we will look at the effects of integrin manipulations upon the Sdc2,4 double morphants. If manipulating the integrins in the Sdc2,4 double morphant yields the same result as integrin manipulations to the 2-OST morphant, that supports the hypothesis that GAG chains on the syndecans are important targets for 2-OST modifications. Furthermore, if manipulating the Sdc2,4 double morphant embryos with an integrin inhibitor results in a loss of the actin mat, this would suggest that integrins are necessary for the integrity and organization of that structure. This would then provide a mechanism for how 2-OST modifications lead to epiboly initiation defects.

In addition to the alterations in the cytoskeletal arrangements, Sdc4 also seems to play a role in modulating FGF signaling as knockdown of Sdc4 alone well as Sdc2,4 together lead to reduced levels of phospho-ERK. This suggests that Sdc4 is more important for modulating FGF signaling than Sdc2. Therefore, Sdc4 may be necessary for FGF ligand interaction with the FGF receptor. Syndecans heterodimerize and homodimerize, suggesting that the Sdc4 homodimer alone or perhaps both the Sdc4 homodimer and Sdc2,4 heterodimer may be necessary for FGF signaling (Choi et al., 2005). Therefore, 2-OST might modulate FGF signaling by affecting the ability of the syndecans to dimerize.

One way to understand how Syndecans and 2-OST modifications are altering FGF signaling and ERK activation may be to co-inject rescue constructs with

modifications to different domains of the Sdcs along with the Sdc MOs. This may allow us to identify how these proteins and GAG chains are modulating the effects upon signaling. For example, if the 2-OST modifications to the GAG chains are preventing the dimerization of the Sdcs, Sdc constructs lacking GAG attachment sites might rescue the phenotype. There are many caveats to the interpretation of these results so carefully considering all the possible reasons for an observed result is necessary. However, further understanding the mechanism behind these signaling alterations will be useful.

Noncanonical Wnt and 2-OST

Many studies have shown that when canonical Wnt signaling is reduced, non-canonical Wnt signaling activity increases. It is unclear whether this is occurring in 2-OST morphants but it remains a possibility. To date no members of the planar cell polarity (PCP) pathway have been shown to play a role in epiboly, but this is possibly due to functional redundancy because it is a very important pathway for development. Furthermore, studies knocking down several components of the PCP pathway simultaneously have not been published but might reveal earlier defects. Several genes hint at a possible PCP pathway involvement in epiboly. The Src kinases Yes and Fyn converge on RhoA to modulate convergent extension (CE) movements and seem to operate in a pathway parallel to Wnt5 and Wnt11. Wnt5 and Wnt11have a characterized role in CE and additionally converge on RhoA too (Jopling and den Hertog, 2005; Zhu et al., 2006). Several other genes which exhibit epiboly defects also converge on RhoA, including Gα12/13 proteins (Lin et al., 2009), Chp/PIX/PAX pathway (Tay et al., 2010), and Diaph2 (Lai et al., 2008), and RhoA is well known for its role in cytoskeletal remodeling. We previously attempted to determine if Src signaling is altered in 2-OST

morphants. The results were difficult to interpret due to high antibody cross-reactivity between the different Src proteins. However, this branch of the signaling cascade may yield valuable results in future studies.

Calcium Signaling May Contribute to the Epiboly Initiation Defect

Calcium signaling is another area that would be a strong target for exploration in the 2-OST morphant phenotype. Several studies have demonstrated calcium signaling is important to many events in development, including cytokinesis and axis establishment (Webb and Miller, 2003, 2007). Directly blocking calcium signaling causes an arrest in epiboly (Cheng et al., 2004). Expression of a dominant negative Fyn kinase results in a failure to initiate epiboly which the authors conclude is due to a defect in calcium signaling (Sharma et al., 2005). Furthermore, some of these genes that exhibit altered calcium signaling also display defects in their cytoskeletal components. In another study, Holloway et al. speculate the rapid actin organization and constriction at the margin in betty boop, a mutant for a kinase in a MAPK cascade, is partly due to a sudden increase in calcium signaling (Holloway et al., 2009).

Further 2-OST MO Studies

The focus of our studies on 2-OST has revolved around epiboly initiation defects. We also created a splice blocking 2-OST morpholino that gives efficient knockdown at fairly low doses based on RT-PCR results. The problems encountered with the splice blocking phenotype led me to move away from those studies. Firstly, the splice blocking morpholino gave a range of later phenotypes, but did not interfere with epiboly. Secondly, we lacked a good antibody against 2-OST which made it difficult to determine

how long maternal protein lingered. RT-PCR results suggested maternal RNA alone was still present through the end of gastrulation. This made it difficult to identify what time window to focus on to narrow down primary versus secondary effects. Lastly, compounded by these timing problems, we were unsure how rapidly the HSPGs on the cell surface turned over. Those issues aside, examining 2-OST function later in development might still yield great information that might have better correlation with development in other model systems. Building on the results from our early developmental studies, the cell cycle defects might be interesting to look at later in development in terms of eye and brain development whereby 2-OST is expressed later in development (Cadwallader and Yost, 2007). Some other preliminary results have suggested that 2-OST may be important in the migration of cells in the primordial heart field (Cam Arrington, personal communication).

Top Hits from Microarray

A microarray study was performed comparing WT embryos to 2-OST morphants at epiboly initiation and at 75% epiboly. The microarray analysis was performed with the aim that some major signaling cascade would emerge which we had not been able to identify previously. However, most signaling pathways were altered by the 2-OST knockdown. Using a high statistical significance (p-value <0.005), approximately 6,000 hits came up. The possible functional relationships between the most dramatically differentially regulated genes and the 2-OST morphant phenotype were not clear. Unexpectedly, most of the top hits were many-fold down in the morphants while very few genes appeared to have significantly increased transcription in the absence of 2-OST. Furthermore, many of the genes have not been characterized or even identified.

Based on the top hits that have been identified some interesting trends emerge. Many of the genes are probably unrelated to the epiboly defect, but may have relevance for other ways 2-O-sulfation contributes to development and disease. For example, several genes related to retinoic acid signaling are down, including Cellular Retinoic Acid Binding Protein 2b, Prostaglandin-endoperoxide synthase 2a (COX-2), and CCAAT/enhancer binding protein (C/EBP) α (Eisinger et al., 2006). It is unclear whether these genes are tied in to the Wnt signaling defect or are independent of the β-catenin defect. Further studies would be needed to establish this. The fact that COX-2 is significantly down is surprising because it is the inducible form of the protein and appears to be expressed at very low levels in WT embryos at this stage in development. Yet several genes involved in its regulation are down too, suggesting this may be a significant piece of data. COX-2 is involved in a variety of activities including prostanoid biosynthesis, inflammation, and tumorigenesis (Morita, 2002; Warner and Mitchell, 2004). COX-2 function also overlaps with other signaling pathways affected by 2-OST knockdown including those mentioned above. However, it is independent of others including regulation of microfilaments, exocytosis, convergent-extension, intermediate signaling kinases, oncogenes, molecular transporters and donors, ECM, and immune system development. Examining how these genes are affected by 2-OST modulation could provide valuable insight to other cellular activities and communication with clinical relevance.

Potential Roles for 2-OST in Other Epiboly-modulating Cascades

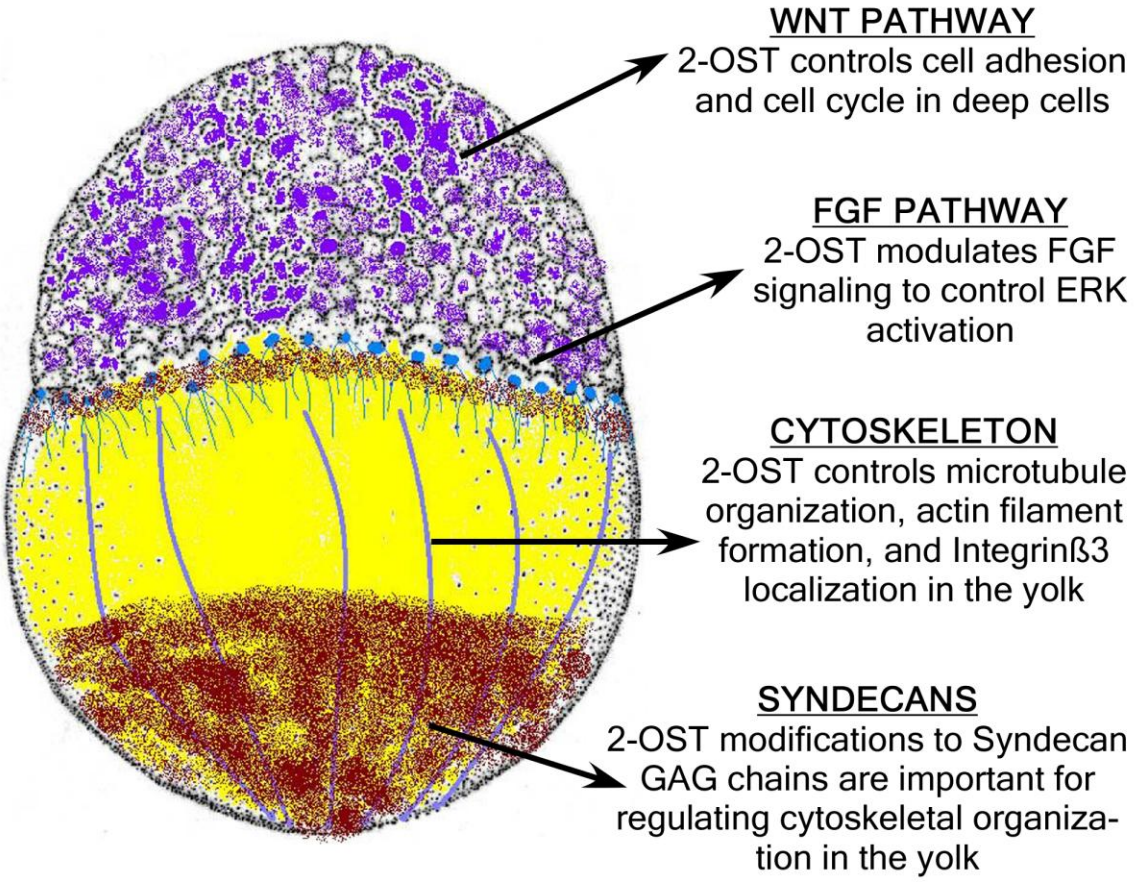
To date about thirty genes are known to play a role in epiboly initiation and progression (Itoh, 2007). We have not yet had the opportunity to examine many of them,

but a few may prove to be of particular interest. The prion-related protein morphant exhibits quite a few phenotypes which are similar to the 2-OST morphant (Edward Malaga-Trillo, personal communication), suggesting there is a possibility 2-OST modifications affect the interactions of that protein on the cell-surface. It might be of interest to examine whether 2-OST morphants modulate membrane microdomain interactions, which also potentially ties in with normal prion protein function (Solis et al., 2010). This may help address the epiboly initiation defect in the 2-OST morphants as well as permitting insight into how 2-OST modifications normally function. Another gene of interest not yet examined in the 2-OST morphants is Profilin 1. Profilin 2-like, a related family member, is shown to be down in the microarray and profilins interact with actin as well as a variety of other proteins (Jockusch et al., 2007). One of these known interactions involves Diaph2 which also contributes to epiboly (Lai et al., 2008), making it a potentially useful target for exploration. Although the microarray analysis did not reveal dramatic changes in the RNA expression for most of the genes implicated in control of epiboly, if 2-OST activity is most important at the cell surface, it may be modulating interactions between the proteins which may not necessarily be reflected in a loss of RNA levels. However, not all the genes which seem to play a role in epiboly regulation were on the microarray chips.

Conclusion

In this dissertation we have demonstrated that 2-OST modifications play a role in controlling of epiboly initiation and progression (Fig. 4.1). It does this in part by controlling multiple signaling cascades. 2-OST activity modulates canonical Wnt signaling, specifically regulating the levels of β-catenin and E-cadherin, which contribute

Figure 4.1. Model summarizing the many roles of 2-OST in epiboly.


WNT PATHWAY
2-OST controls cell adhesion and cell cycle in deep cells
FGF PATHWAY
2-OST modulates FGF signaling to control ERK activation
CYTOSKELETON
2-OST controls microtubule organization, actin filament formation, and Integrinß3 localization in the yolk
SYNDECANS
2-OST modifications to Syndecan GAG chains are important for regulating cytoskeletal organization in the yolk

to the regulation of cell adhesion and proliferation. We have also demonstrated that 2-OST activity plays a role in regulating FGF signaling and cytoskeletal remodeling. Therefore, 2-OST activity modulates a variety of signaling pathways and cell behaviors, resulting in a compound phenotype, suggesting 2-O-sulfation is important for a wide range of cellular communication.

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APPENDIX

ZINC FINGER NUCLEASES

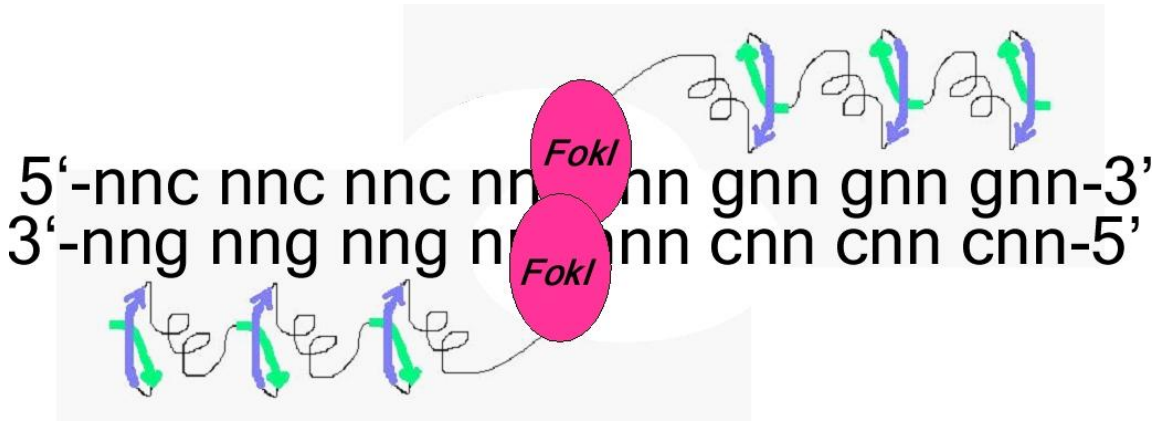
Introduction

Over the last few decades, *Danio rerio* has become a popular model system due to the many advantages offered over other vertebrate models. They produce a large number of progeny in each clutch, they are externally fertilized and transparent making it easy to genetically manipulate them during early development via sense and antisense technology. They also bear greater homology to humans than invertebrate model systems, making them ideal for modeling development and disease. However, one of the drawbacks in working with zebrafish is the lack of a method to create efficient targeted mutant populations. Genes can be knocked down transiently with morpholinos, but the stability of morpholinos only lasts a few days. Vectors and retroviruses create random insertion mutants, chemical mutagenesis creates mutants that can be identified via positional cloning (Amsterdam and Hopkins, 2004; Wienholds and Plasterk, 2004), and transgenic fish can be created with transposable elements, random plasmid insertions, and BAC vectors (Culp et al., 1991; Shin et al., 2003; Balciunas et al., 2004). However all of these techniques lack specificity, which is one of the reasons why gene targeted knockdown using cre-lox system part of what made the mouse popular for genetic manipulation studies (Capecchi, 1989).

To circumvent these problems, we attempted to develop a technology for zebrafish which has previously been successful in *Xenopus*, *Drosophila*, cell culture, *Arabidopsis*, and *C. elegans* (Segal and Carroll, 1995; Bibikova et al., 2002; Porteus and Baltimore, 2003; Lloyd et al., 2005; Urnov et al., 2005). This approach induces targeted double-strand breaks in DNA and then exploits the repair mechanisms endogenous to the cell to promote the introduction of new mutations. When breaks occur in DNA, two mechanisms are used to repair them; nonhomologous end joining (NHEJ) which may introduce new mutations, or homologous recombination (HR) which should be identical to the sequence before the break (Hagmann et al., 1998; Bladen et al., 2005). This allows introduction of either novel mutations by NHEJ in our gene of interest, or insertion of a new sequence by co-injecting a plasmid to be incorporated in via homologous recombination.

This technique works by designing zinc fingers proteins which recognize highly specific sequences of 9 DNA base pairs in length (Kim et al., 1996). These zinc fingers are connected to a nuclease domain which needs to dimerize in order to be functional. Therefore, two sets of these zinc fingers are designed to flank a region that is six base pairs in length, allowing the zinc fingers to bind upstream and downstream of this region so that the nuclease Fok1can dimerize and cleave the DNA at this specific location (Fig. A.1) (Smith et al., 2000). The first goal of this project was to develop a system for site directed mutagenesis (SDM) induced by NHEJ using zinc finger nucleases (ZFN) in zebrafish. The next goal was to be able to create germ line mutants to demonstrate that this SDM is heritable. Lastly, we wanted to use this approach to develop a system for

Figure A.1. Model of ZFN. The triplet zinc fingers recognize three base pair sequences on either side of the 6-base pair spanning region. The FokI nuclease dimerizes to induct cleavage in the 6-base pair spanning region.


FokI
FokI
5‘-nnc nnc nnc nn nn gnn gnn gnn-3’
3‘-nng nng nng n nn cnn cnn cnn-5’

targeted gene replacement via HR in a specific sequence using zinc finger nucleases and a modified sequence in a donor vector.

Results and Discussion

Synthetic Target

Our first step was a proof of principle experiment to determine if this system would be functional in zebrafish. We wanted to determine if injected RNA encoding the zinc fingers and nuclease could be expressed. Furthermore we wanted to know if these expressed proteins would be functional. The synthetic target was a palindromic sequence known as QEQ with fingers which would recognize the same sequence on the top and bottom strand with a restriction enzyme site between the two (Fig. A.2A). We performed Western blots to determine whether or not the protein was being expressed. Initially none of the buffers we conventionally use in the lab worked. However implementing modified buffers based on Fok1 westerns in flies we were able to visualize the levels of protein via Western blot (Fig. A.2B). This showed that we were able to translate the zinc fingers and Fok1 in fish. This gave us a sense of the range of RNA to inject to induce cleavage in the synthetic target.

Next, we tested whether we could actually induce cleavage. The embryos were injected with ZFN RNA and the synthetic QEQ target. Individual embryos were collected and lysed to extract DNA. Primers were designed flanking the target site and the sequence was amplified using PCR. The PCR products were then digested with *EcoRI* and run out on an agarose gel. The loss of the EcoR1 restriction site was used as an indication of cleavage by Fok1 and repair by NHEJ. An unmodified product would yield three bands while a modified product which lost the restriction site would have two

bands (Fig. A.2C). We were able to induce NHEJ cleavage and repair in most embryos (Fig. A.2D). However injecting a constant amount of RNA and plasmid resulted in a range of digestion efficiency suggesting a range of cleavage occurred in each embryo. To determine the types of repair that occurred at these breaks, we subcloned some of the PCR products which produced positive hits (Fig. A.2E) and found both insertions of random base pairs as well as deletions.

Endogenous Targets

After proving we could get the system to work using artificial targets, the next stage was to identify gene targets which met several criteria. To rapidly screen for successful cleavage and repair the first priority was to identify a target with a restriction nuclease sequence in those six base pairs where the Fok1 should cleave so if NHEJ occurred, the restriction nuclease should be unable to recognize its target sequence. Secondly, we wanted the mutated gene to provide viable progeny so that we could use it for germ line screens. Third, we had very specific sequence parameters to engineer zinc fingers which should bind the DNA with high efficiency based on the following sequence pattern: 5'-*nn*c *nn*c *nn*c *nnnnnn* g*nn* g*nn* g*nn*-3'. Lastly, we wanted to try to find these elements in our genes of interest. We screened 46 genes and found nearly half had at least one site that met these criteria (Fig. A.3A).

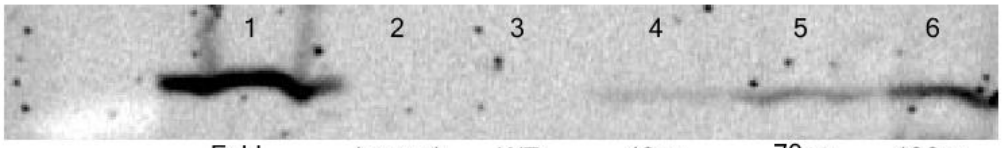
Of the 46 possible gene targets, we selected two: *fms* and *lefty2*. *Fms* was selected because it has a very obvious mutant phenotype, a loss of pigmentation which results in spots instead of stripes (Parichy and Turner, 2003). *Lefty2* was selected because it contributes to organ asymmetry but is not necessary for viability (Bisgrove et al., 2000). We selected one site in *fms* and two sites in *lefty2* and engineered ZFN for

Figure A.2. Synthetic target for ZFN. QEQ synthetic target sequence with EcoRI restriction site between the two binding sites (A). Western blot results demonstrating that increased concentrations of ZFN RNA injected results in increased protein expression, comparing levels of 40 pg, 70 pg, and 100 pg RNA. Purified FokI protein was used as a control for antibody function (B). Diagram comparing the expected fragment lengths for unmodified QEQ sequence and the modified QEQ sequence resulting in the loss of the *EcoRI* restriction site (C). PCR digestion results showing embryo per lane with ZFN RNA but no QEQ target DNA (negative control, lanes 1-3), QEQ target DNA but no ZFN RNA (positive control, lanes 4-6), and embryos co-injected with 70 pg ZFN RNA and QEQ target DNA (lanes 7-18) showing variable levels of efficiency (D). Subcloned sequences showing different modifications to the QEQ target, including deletions, insertions, and concatemers (E).

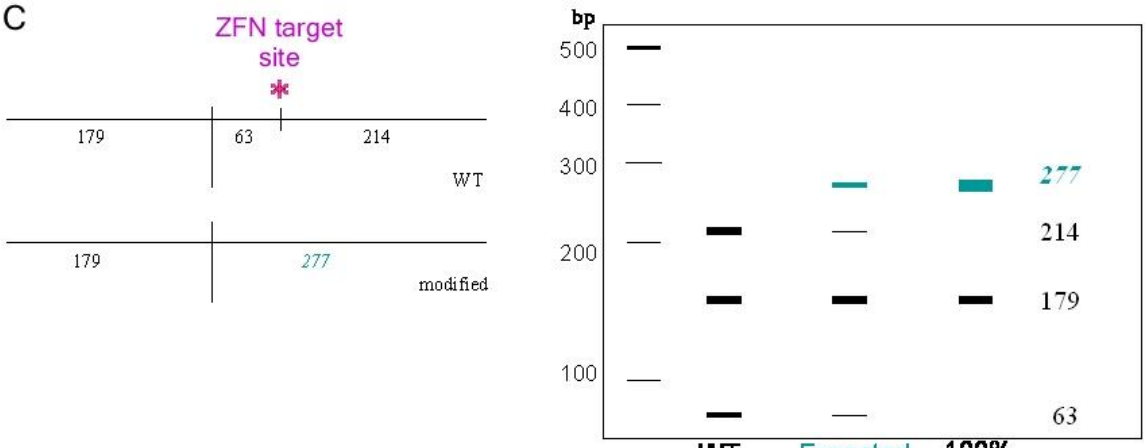
A

5'-TTC TTC CCC *GAATTC* GGG GAA GAA-3'
3'-AAG AAG GGG *CTTAAG* CCC CTT CTT-5'
EcoRI site

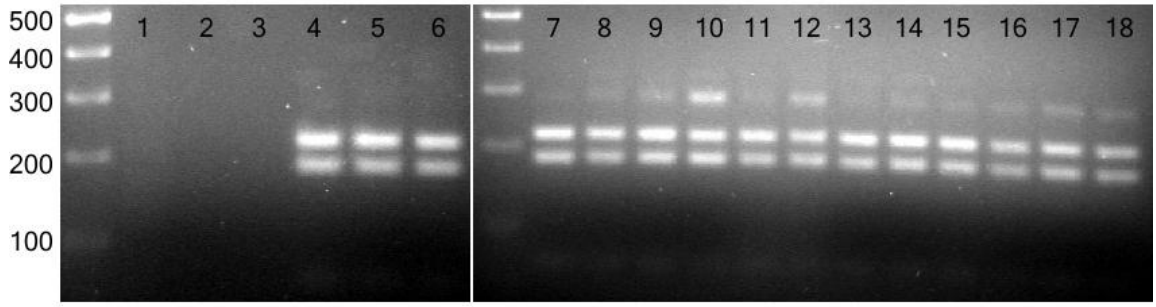
B



C



D



E

QEQ: GGTACCTTCTTCCCCGAATTCGGGGAAGAA
8: GGTA::::::::::::::::::::::::::::::::ATTCGGGGAAGAA (13 bp deletion)
11: GGTACCTTCTTCCCCGAATT*T*CGGGGAAGAA (1 bp insertion)
12: GGTACCTT::::::::::::::::::::AATTCGGGGAAGAA (8 bp deletion)
19: GGTACCTTCTTCCCCG*GGTTAGGGTTAGGGTTA*GGGGAAGAA
(insertion of GGGTTA concatemer)

these targets. The only finger combination which seemed to work was the first set of *lefty2* fingers (Fig. A.3B). The efficiency of these cuts was quite low per embryo but it was detectable (Fig. A.3C). Despite repeated attempts at subcloning the populations where there was a loss of the restriction site, we were only able to successfully clone one which showed a several base pair deletion (Fig. A.3D). We terminated the project before I finished plans to optimize the other fingers or before we grew any up to look at germline transmission.

Conclusion

This project was terminated as my thesis project for several reasons, the most important was that I was getting scooped and would have nothing towards a publication for my dissertation (Meng et al., 2008). Furthermore, the published paper used a yeast two hybrid system that allowed them to screen multiple combinations of fingers to identify the most efficient pairs, a system far more efficient than our own. However the two years spent on this project were not a total loss. I learned a lot of valuable lessons about experimental design, the importance of controls, and troubleshooting problems with Westerns and PCR. Tackling the challenges this project presented was an enjoyable experience. I also gained experience writing grants even though I didn't receive funding for them.

Materials and Methods

Zinc Finger Construction

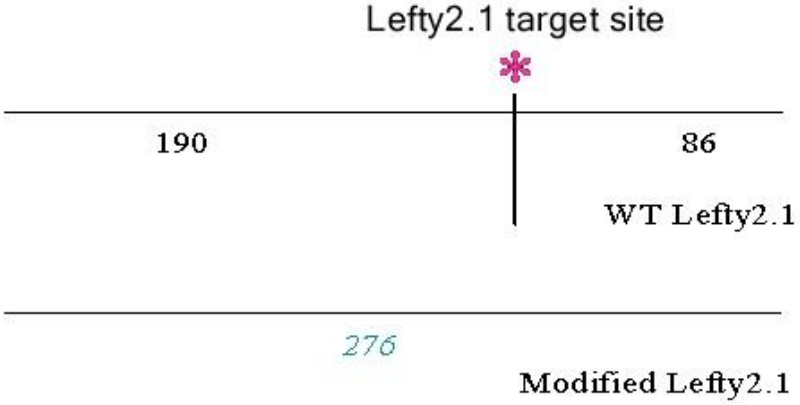
The targets were identified and fingers were designed using a previously established set of fingers. Long overlapping primers were designed to create the fingers

Figure A.3. Endogenous targets for ZFN. After screening a total of 46 genes for potential target sequences, we charted the distribution of potential targets in these genes, so 12 genes had no target sites, 14 had one target site, etc. (A). A model of the unmodified PCR product for Lefty2.1 cleaved with HinPI and the modified product (B). An agarose gel with an uncut control band (lane 1) and several selected samples that were redigested to confirm that some faint band at 276 bp was visible (C). These bands were cut out and subcloned, but only one vector gave us a clone which showed an 8 bp deletion (D).

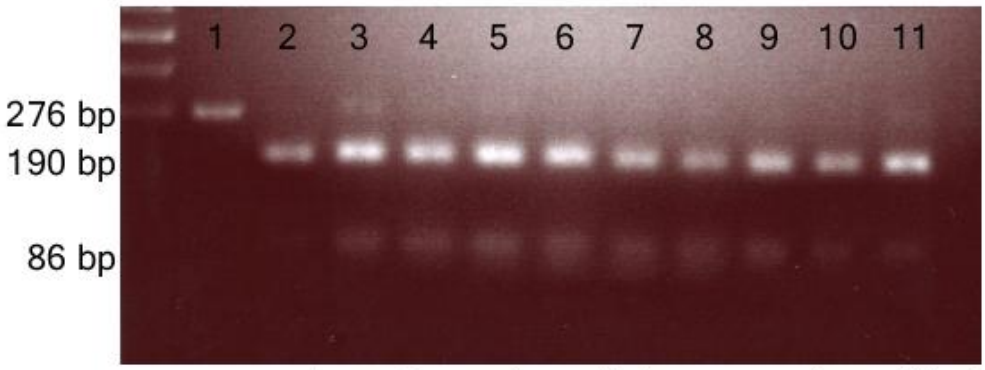
A

Target Sites	# of Genes
0	12
1	14
2	6
3	3
4	6
5	2
6	2
24	1
TOTAL	46

B



C



D

WT: AGCTCCATCACCAGCGCAGGAGGAGATC
33: AGCTCCATCA.....GGAGGAGATC (8 bp deletion)

via PCR and were cloned into compatible vectors for transcription. RNA was transcribed using Message Machine Kits and injected into the embryos.

Western Blots

The recipe for the extraction buffer which proved to most effectively denature the FokI protein was 100 mM Tris (pH 6.8), 8% SDS, 20% glycerol, .5x phosphatase inhibitor, 2x protease inhibitor, 20% BME, and bromophenol blue to taste.

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