RESEARCH STRATEGY

SIGNIFICANCE

Summary: The study of the secreted peptide Toddler as described here will benefit developmental biology and human health. First, these aims will increase our understanding of the molecular and cellular basis of gastrulation, a process fundamental to all animals. Second, the peptide Apelin, which signals through the same GPCRs as Toddler, is currently being studied for treatment of cardiovascular diseases and studying its interaction with Toddler will supplement our understanding of this putuative drug target (Charles 2011).

There is a missing link during gastrulation.

Early in development an epithelial layer of cells, called the enveloping layer (EVL), covers an indistinct group of cells called the blastocyst. During gastrulation, the cells of the blastocyst and the EVL move over the yolk toward the vegetal pole in a process called epiboly. During epiboly, some of the blastocyst cells internalize at the margin and form a new, distinct, hypoblast/mesendodermal layer, which will go on to become endoderm or mesoderm and migrate animally. The exterior layer, which continues to migrate vegetally, is the epiblast and will become ectoderm (Figure 1)(Solnica-Krezel and Sepich 2012). It is known that Nodal, a member of the TGF-ß superfamily, is an essential signal for the specification of mesendoderm (Schier and Talbot 2005). Fate mapping studies in Nodal mutants have shown that mesendodermal progenitors in the margin respond differently to Nodal based on their location along the animal-vegetal axis and in a dosage dependent manner (Dougan, Warga et al. 2003; Schier 2009). For example, cells will acquire endodermal (high Nodal), mesodermal (medium Nodal) or ectodermal (low Nodal) fate. Though this pattern formation during blastula stages of development is relatively well understood, how it connects to subsequent gastrulation movements is still unclear. Moreover, while research to date suggests that Wnt, P13K and other pathways modulate gastrulation through cell adhesion and migration, the roles of the individual pathways, their mechanisms and how they work together to regulate gastrulation is unknown (Rohde and Heisenberg 2007).

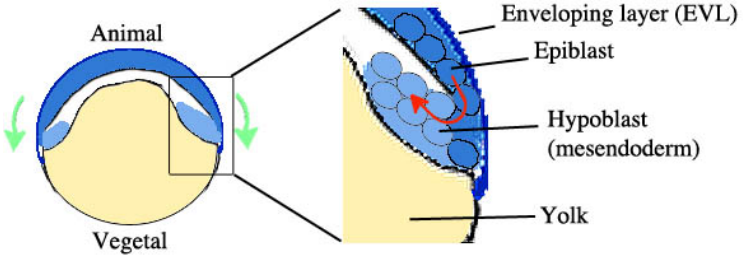

Figure 1. Overview of cellular movements during gastrulation. (a) Cartoon lateral cross section of an embryo at 50% epiboly. Dorsal is to the right. Green arrows show direction of epiboly and epiblast migration. (b) Close up of boxed region in (a). Dark blue is EVL, blue is epiblast, light blue is hypoblast. Red arrow indicates direction of hypoblast internalization and migration.

Toddler is a novel signal active in gastrulation.

Recently, the Schier lab discovered a highly conserved and novel gene, *toddler*, which is active and required during zebrafish gastrulation (Figure 2c). Differently from wildtype, endoderm and mesoderm in *toddler* mutants fail to properly migrate animally (Figure 2a and b), raising the hypothesis that this gene may help link blastula and gastrula stages of development and/or explain the effector mechanism of the known gastrulation pathways. Loss of Toddler causes a decrease in the speed of endodermal cell movements, the number of cells that move anteriorly and the ability of cells to completely transition from epiblast to hypoblast. Furthermore, the Schier lab has shown that Toddler is a small secreted peptide, signals through the Apelin receptors A and B (AplnrA/B) and is required to survive past 7 days post fertilization (dpf) in

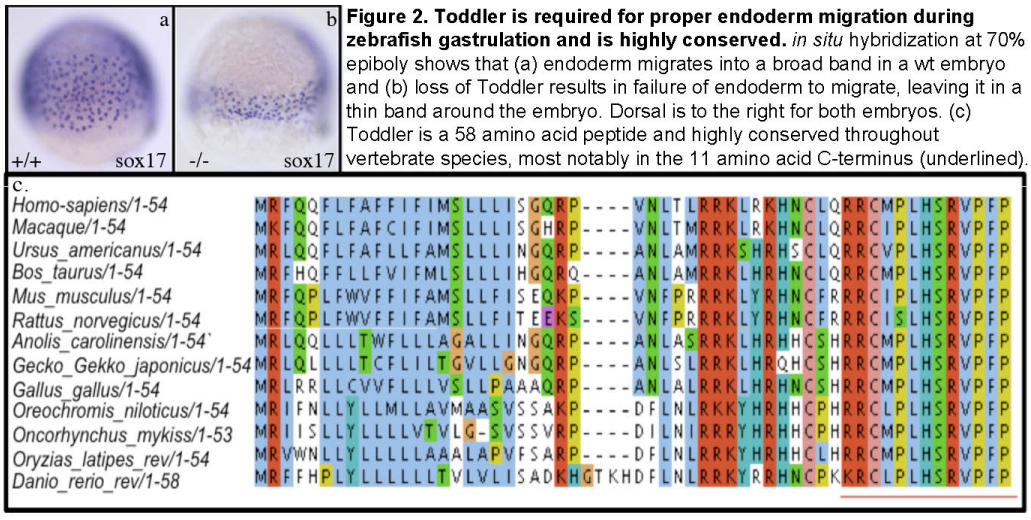

Figure 2. Toddler is required for proper endoderm migration during zebrafish gastrulation and is highly conserved. *in situ* hybridization at 70% epiboly shows that (a) endoderm migrates into a broad band in a wt embryo and (b) loss of Toddler results in failure of endoderm to migrate, leaving it in a thin band around the embryo. Dorsal is to the right for both embryos. (c) Toddler is a 58 amino acid peptide and highly conserved throughout vertebrate species, most notably in the 11 amino acid C-terminus (underlined).

zebrafish. *Toddler* mutants have significant morphological defects, most noticeably a small or absent heart and posterior accumulation of blood (Pauli, Norris et al. In review).

APPROACH

Previous and preliminary studies

Summary: my graduate studies to date have assisted in characterization, both qualitatively and quantitatively, of the *toddler* mutant phenotype (Pauli, Norris et al. In review).

Toddler promotes cell movement

Work in the Schier lab carried out by Dr. Andrea Pauli, myself and others led to the discovery and characterization of Toddler (Pauli, Norris, Valen, Chew et al. In review). This work identified Toddler as a novel signal required during early embryogenesis and used genetic and embryologic techniques as well as microscopy to demonstrate that Toddler is a processed and secreted modulator of cell migration during gastrulation. Using *in situ* hybridization analysis of multiple stages and reporter genes, my work demonstrated that both mesoderm and endoderm are defective in moving over the embryo in *toddler* mutants. Dr. Pauli used lightsheet microscopy to assess the possible reasons for these migratory defects and demonstrated that loss of Toddler causes a decrease in migrating cell speed as well as defects in cell internalization and movement animally, as compared to wildtype.

Toddler signals via Apelin Receptors A and B

Previous research has shown that the AplnrA/B are active beginning at 5 hpf, which is approximately when Toddler is first expressed (Scott, Masri et al. 2007). Furthermore, AplnrA/B knockdown embryos are reported to have heart defects (Scott, Masri et al. 2007; Zeng, Wilm et al. 2007). To confirm that Toddler is signaling through AplnrA/B, Dr. Pauli used confocal microscopy and fluorescently labeled receptors to show that the receptors are internalized in response to Toddler signaling. Additionally, I used *in situ* hybridization to demonstrate that knockdown of AplnrA/B causes the same mesendoderm migratory defects as observed in *toddler* mutants.

In summary, it has become clear through my preliminary work with Dr. Pauli and others in the lab that the conserved peptide Toddler is an important signal in vertebrate development. The degree of conservation is evident in the multiple sequence alignment (Figure 2c) and further underscored by the observation that over-expression of human Toddler in zebrafish mimics overexpression of the zebrafish peptide (Figure 3). However, the cellular behavior affected (Aim 1), the molecular mechanism (Aim 2), and an understanding of how Toddler complements the Apelin receptor pathway (Aim 3) is yet to be elucidated.

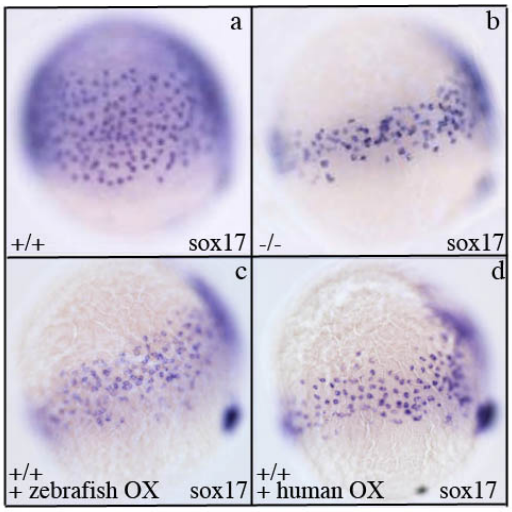

Figure 3. Overexpression of Toddler mRNA from human or zebrafish in zebrafish embryos phenocopies a Toddler mutant. *in situ* hybridization for sox17 at 70% epiboly shows mutants (b) have a defect in endoderm migration as compared to wt (a). Overexpression of zebrafish (c) or human (d) transcripts of Toddler cause similar defects in endoderm migration.

Experimental Design

Aim 1: Characterize the behavior of Toddler-responsive cells as they migrate.

This aim will generate genetic reporters to label Toddler-responsive cells (Aim 1.1), determine what leads to the decrease in the number of internalized cells in *toddler* mutants (Aim 1.2) and address the hypothesis that Toddler affects a single germ layer during gastrulation (Aim 1.3).

Aim 1.1 Generate genetic reporters for live analysis.

To assess the behavior of AplnrA/B-expressing/Toddler-responsive cells directly without requiring the use of indirect analysis tools such as endodermal and mesodermal reporter lines, I will generate AplnrA and AplnrB reporters by two complimentary approaches. In one, I will utilize bacterial artificial chromosome (BAC) recombineering, which is the traditional method for mimicking endogenous protein expression in zebrafish (Suster, Abe et al. 2011). BACs contain large portions of zebrafish genomic DNA and can be manipulated to express fluorescent proteins driven by the regulators of a given gene, thereby recapitulating endogenous expression of that gene. The recombineered BAC, which also contains long terminal repeats of the Tol2

transposon, is then integrated into the zebrafish genome, generating a stable transgenic line. For the second method, I will use obligate ligation-gated recombination (ObLiGaRe) to integrate a fluorescent protein directly into the endogenous genomic loci by nonhomologous end joining (Maresca, Lin et al. 2013). To this end, I will use Crispr-led Cas9 endonuclease to cleave site-specifically at the *aplnrA* and *aplnrB* loci (Auer, Duroure et al. 2013). The Crispr/Cas9 system is a genome-editing tool driven by a short RNA (gRNA) that targets Cas9 endonuclease to a region of interest (Hwang, Fu et al. 2013).

Aim 1.2 Determine what behavior leads to fewer internalizing cells in toddler mutants.

Previous work has demonstrated that fewer cells internalize in *toddler* mutants (Pauli, Norris et al. In review). However, limitations in previous experiments made Toddler-responsive cells indistinguishable from Toddler-nonresponsive cells. As such, it is unclear how Toddler signaling directly affects internalization behavior. This raises two main questions. First: How many of the internalizing cells in an embryo express AplnrA/B and are thus Toddler-responsive? Second, how does loss of Toddler cause fewer of these cells to internalize? To address these questions I will use my AplnrA/B reporter lines to monitor the number and behavior of these cells during gastrulation. First, I will address the fact that it is not currently known what percentage of internalizing cells express AplnrA/B. I will inject the AplnrA/B reporter lines with histone-RFP mRNA to label all cell nuclei and use lightsheet microscopy and cell tracking to quantify the number of internalizing cells that are also expressing the receptors in wildtype embryos. If all internalizing cells express the receptors, it would suggest Toddler may be a major internalization cue. Alternatively, it is possible that only a subset of internalizing cells express the receptors, suggesting Toddler signaling through AplnrA/B is not a universal internalization requirement. Knowing the proportion of cells that express AplnrA/B and are thus capable of responding to Toddler will allow me to then ask why fewer are seen internalizing in *toddler* mutants. There will be two main possibilities: either there are fewer AplnrA/B cells present in the embryo or the cells are present yet fail to internalize. To determine which cause is at play, I will first count the total amount of AplnrA/B-expressing cells in wildtype and *toddler* mutant embryos. Next, I will track AplnrA/B-expressing cells during gastrulation and compare the relative amount of internalizing and noninternalizing cells between wildtype and mutants. I will then compare the changes in relative cell number and internalization amounts to determine what degree of the internalization phenotype can be explained by either scenario. A decrease in the total number of AplnrA/B-expressing cells would suggest that Toddler is required in some way to maintain the AplnrA/B-expressing cell type while an over abundance of AplnrA/B-expressing cells failing to internalize would suggest that Toddler is a necessary signal for their internalization.

Aim 1.3 Determine which germ layers are directly affected by Toddler signaling.

Both endoderm and mesoderm are specified during internalization and both germ layers are affected in their animal-directed movements in *toddler* mutant embryos (Pauli, Norris et al. In review), yet it is unclear if Toddler signaling directly affects both layers. I hypothesize that one layer is the primary target of Toddler and its defect leads to secondary phenotypes in the other layer. To directly test this hypothesis I will cross AplnrA/B reporter lines with endoderm or mesoderm reporter lines, sox17::eGFP or drl::eGFP respectively, to analyze the expression overlap after germ layer specification in wildtype embryos. This will reveal whether AplnrA/B-expressing cells are exclusively present in one germ layer or remain in both endoderm and mesoderm after their differentiation from mesendoderm. As AplnrA/B are reported to be mesodermal, I expect that AplnrA/B will become isolated to mesodermal cells after internalization and specification (Figure 5) (Zeng, Wilm et al. 2007). If I find that endoderm does not express AplnrA/B it suggests there is an indirect effect of Toddler signaling, via mesoderm, on the endodermal germ layer. To formally test this, I will carry out transplantation experiments followed by cell tracking. First I will transfer either mesodermal or endodermal cells from AplnrA/B knockdown embryos into wildtype embryos. If Toddler signaling directly affects mesoderm and not endoderm, the transplanted mesodermal cells should fail to migrate, while the endodermal cells should appear wildtype. If Toddler indirectly affects endoderm via the mesoderm, I should still see wildtype behavior of the transplanted endoderm because the host mesoderm is wildtype. Next, I will do the converse experiment and transfer wildtype mesoderm or endoderm into AplnrA/B knockdown hosts. If Toddler only acts on mesoderm, the transplanted mesodermal and endodermal cells should migrate normally. However, if Toddler signaling is indirectly needed for endoderm movements, the transplanted wildtype cells will not migrate normally due to the absence of Toddler signaling in the host mesoderm.

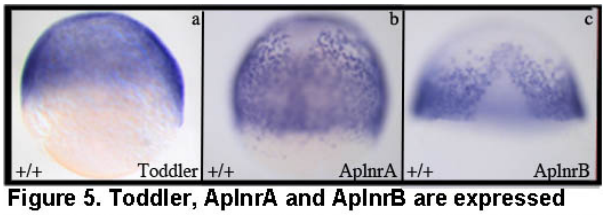

Figure 5. Toddler, AplnrA and AplnrB are expressed during zebrafish gastrulation. *in situ* hybridization at 70% epiboly shows that Toddler is expressed ubiquitously (a), and AplnrA/B (b,c) are restricted to to the hypoblast.

Possible limitations

I do not foresee any technical limitations for the BAC transgenesis, as the Schier lab has used this technique extensively (Huang, Xiong et al. 2012). Generating the genome-engineered reporters will be challenging, but current work in the lab has successfully employed the Crispr/Cas9 system for the mutagenesis of >100 genes in zebrafish and furthermore demonstrates the use and success of Obligare. I will work alongside other members as we utilize and adapt Obligare to our individual genes and needs. Microscopic analyses, including more recently lightsheet microscopy, are frequently used in the Schier lab for a range of applications (Muller, Rogers et al. 2012; Pauli, Norris et al. In review). As such I am in the ideal location for characterizing Toddler's affect on cellular behavior during development through live-cell imaging. Transplantation experiments are a common experimental technique within the Schier lab and I do not anticipate any major obstacles in utilizing this technique thanks to the aid of experienced fellow lab members. However, the ability to isolate only endoderm or only mesoderm as is required in these experiments may be challenging. It is possible to manipulate embryos to produce only endoderm, simplifying the process of specific endoderm transplantation. Specific transplantation of only mesoderm will require extreme precision and thus may require extensive practice in the technique before the experiment can be carried out rigorously.

Aim 2: Study the role of Toddler in E-cadherin dependent cell adhesion.

Loss of Toddler decreases motility and appears to increase cell clumping. Like Toddler, cell adhesion is dosage dependent, with proper migration requiring neither too little nor too many adhesion molecules (Schwartz and Horwitz 2006). These observations lead me to hypothesize a role for Toddler in regulation of cell adhesion. To address this, I will test whether altered E-cadherin, known to play a role in gastrulation, contributes to the *toddler* mutant phenotype (Shimizu, Yabe et al. 2005; Rohde and Heisenberg 2007).

Aim 2.1 Determine if Toddler positively or negatively regulates E-cadherin expression and membrane localization.

I will first compare and quantify the amount of E-cadherin protein in wildtype and *toddler* mutant embryos by Western blot to survey whether E-cadherin expression levels are affected. However, expression level is not the only way that Toddler may be regulating E-cadherin and it is possible that though the relative abundance of E-cadherin is the same, its presence and uniformity at the membrane may be affected. Thus, I will secondly analyze changes in localization of the cellular adhesion machinery in wildtype and *toddler* mutant embryos throughout early development through immunohistochemistry with antibodies against E-cadherin and beta catenin, which is part of the linker between E-cadherin and the cytoskeleton (Shimizu, Yabe et al. 2005). Toddler regulation of E-cadherin would present three possible outcomes for this experiment: (1) Toddler may cause increased localization of E-cadherin and beta-catenin at the cell membrane as compared to wildtype. (2) Toddler may cause a decrease of localization at the membrane or (3) there may be changes in the localization of adhesion within the membrane. For example, the pattern may change from equal expression around the cell to preferential junctions with certain neighbors.

Aim 2.2 Determine if reduction or increase in E-cadherin levels can rescue the toddler mutant phenotype.

I will use E-cadherin morpholinos, which inhibit gene specific translation, and mRNA to down regulate or overexpress, respectively, E-cadherin in *toddler* mutant embryos and monitor the developmental phenotypes and motility of cells using *in situ* and *in vivo* analysis. Although this final experiment will be complicated by the fact that E-cadherin is broadly used in development, diligent use of controls, such as extensive analysis of E-cadherin knockdown embryos, and the results of the first two experiments will allow for careful deciphering of E-cadherin's role in the *toddler* phenotype. Specifically, if loss of Toddler causes over abundance in cell adhesion, I should be able to at least somewhat abrogate this phenotype with knockdown of E-cadherin. Conversely, if loss of Toddler causes an under abundance of cell-adhesion, supplementing embryos with increased E-cadherin may be able to rescue the Toddler deficiencies. Completion of this aim will directly address the mechanistic activity of Toddler by elucidating whether the Toddler phenotype is E-cadherin dependent. If it is, it supports the hypothesis that Toddler is active in cell adhesion and allows for more in depth studies of the Toddler and E-cadherin regulation relationship.

Possible limitations

If E-cadherin does not seem to be associated with the Toddler phenotype it could be that manipulation of E-cadherin results in a severe phenotype that masks any role of Toddler, that E-cadherin is not the only cell-adhesion molecule at play, or that E-cadherin is not involved in the Toddler mechanism. To pick apart these possibilities I will turn to *ex vivo* adhesive assays such as adhesion molecule coated wells and cell dissociation and reaggregation assays to directly address changes in adhesive ability in *toddler* mutant cells before

addressing other cell-adhesion molecules in a similar experimental set up (Speirs, Jernigan et al. 2010; Song, Eckerle et al. 2013).

Aim 3: Examine the relationship between Toddler and Apelin in development.

The fact that the Toddler phenotype is variable and can be rescued by exogenously provided Apelin suggests overlap or redundancy in the Toddler and Apelin pathways (Pauli, Norris et al. In review). Currently, however, this is not testable as there are no available *apelin* mutants and *apelin* morpholinos appear to cause non-specific defects.

Aim 3.1 Determine the similarities between apelin and toddler mutant phenotypes.

I will use the Crispr/Cas9 system, commonly used for mutagenesis due to its tendency to cause small indels, to generate an *apelin* mutant in zebrafish (Hwang, Fu et al. 2013). I will use this mutant to compare *toddler* and *apelin* mutant phenotypes, their rescuing capabilities and their functional redundancy. I will begin with a thorough characterization of the developmental phenotypes of *apelin* mutants by *in situ* analysis of reporter genes and examination of larval morphology during early and late development. As Apelin is not expressed until after gastrulation, I expect that the early endoderm and mesoderm migration defects and the later morphological endoderm defects that likely stem from the gastrulation defects in *toddler* mutants will be absent in the *apelin* mutants. However, I anticipate heart formation and vasculature will be affected in *apelin* mutants, although I cannot guess how the severity of the defects will compare to *toddler* mutants.

Aim 3.2 Determine the ability of Toddler to rescue Apelin deficiencies.

Secondly, I will test if Toddler can rescue Apelin deficiencies by injecting *toddler* mRNA into *apelin* mutants. To avoid overexpression of Toddler, thus masking any rescuing capabilities, I will utilize a heat shock inducible *toddler* transgene to only provide exogenous Toddler when Apelin is normally expressed, 10 hours post fertilization and later (Pauli, Norris et al. In review). Though ubiquitous exogenous Toddler and Apelin, at the appropriate levels, can rescue a *toddler* mutant (Pauli, Norris et al. In review), Apelin may have a much more specific domain of action that might be sensitive to changes in expression pattern. If this seems to be the case and ubiquitous Toddler does not rescue, I will turn to BAC recombineering and replace the Apelin ORF with Toddler. After integration into the genome, I will cross this line with my *apelin* mutants to generate a line of fish that express Toddler instead of Apelin under Apelin's endogenous expression pattern. Inability of exogenous Toddler to rescue in this case, though it is restricted to Apelin's specific expression domain, may suggest that one of the ligands is only a partial agonist and/or that there are specific stimulation requirements of the receptors for different stages in development, a common characteristic of GPCRs. Conversely, rescue with the use of BACs would suggest that the ligands are redundant if expressed at the same time and used by the receptors in preference for their pattern of expression.

Aim 3.3 Determine the degree of overlap in the endogenous Apelin and Toddler pathways.

The ability of Toddler and Apelin to rescue or not rescue will not directly address the endogenous activity of the peptides. To discern the actual crosstalk/redundancy in the pathways, I will cross *toddler* and *apelin* mutant lines to generate double mutants. I hypothesize that Apelin signaling just after gastrulation is what allows the Toddler phenotype to be variable. I will use the same characterization tools of morphology and reporter gene expression pattern to qualitatively and quantitatively characterize the double mutant phenotype and relate it to the single mutants. Redundancy and partial redundancy should result in a synergistic phenotype in the double mutant and repress the variability of *toddler* mutants. Conversely, largely non-overlapping roles would result in an additive phenotype and not greatly affect the *toddler* phenotype and variability. Completion of this aim will therefore dissect the genetic crosstalk within this pathway(s) during development and enhance our ability to understand the independent and overlapping Toddler and Apelin phenotypes and mechanisms.

Possible limitations

I do not expect any major difficulties with generation of the *apelin* mutant as the Crispr/Cas9 system has already been used extensively within the lab to generate mutants. It is likely that Apelin is required for maintenance of the organism in addition to its role in development. As such, I will limit my analysis to early development, 36 hours post fertilization and less, to allow me to focus on the early requirements for Apelin without the confounding results of its requirement later in life.