

# *Ilyanassa* Notch signaling implicated in dynamic signaling between all three germ layers

MAEY GHARBIAN<sup>1</sup>, AYAKI NAKAMOTO<sup>1</sup>, ADAM B. JOHNSON<sup>2</sup>, J. DAVID LAMBERT<sup>2</sup> and LISA M. NAGY<sup>1\*</sup>

<sup>1</sup>Department of Molecular and Cellular Biology, University of Arizona, Tucson, Arizona, USA and

<sup>2</sup>Department of Biology, University of Rochester, NY, USA

**ABSTRACT** Two cells (3D and 4d) in the mud snail *Ilyanassa obsoleta* function to induce proper cell fate. In this study, we provide support for the hypothesis that Notch signaling in *Ilyanassa obsoleta* functions in inductive signaling at multiple developmental stages. The expression patterns of Notch, Delta and Suppressor of Hairless (SuH) are consistent with a function for Notch signaling in endoderm formation, the function of 3D/4d and the sublineages of 4d. Veligers treated with DAPT show a range of defects that include a loss of endodermal structures, and varying degrees of loss of targets of 4d inductive signaling. Veligers that result from injection of *Ilyanassa* Delta siRNAi in general mimic the defects observed in the DAPT treated larvae. The most severe DAPT phenotypes mimic early ablations of 4d. However, the early specification of 4d itself appears normal and MAPK activation in both 3D/4d and the micromeres, which are known to activate MAPK as a result of 3D/4d induction, are normal in DAPT treated larvae. Treating larvae at successively later timepoints with DAPT suggests that Notch/Delta signaling is not only required during early 4d inductive signaling, but during subsequent stages of cell fate determination as well. Based on our results, combined with previous reports implicating the endoderm in maintaining induced fate specification in *Ilyanassa*, we propose a speculative model that Notch signaling is required to specify endoderm fates and 4d sublineages, as well as to maintain cell fates induced by 4d.

**KEY WORDS:** *Notch, Delta, mollusc, endoderm, 4d*

## Introduction

In most metazoan animals, establishing cell fates along embryonic axes involves an interplay between asymmetric inheritance and cell signaling. By contrast, specification of the dorsal-ventral axis in *Ilyanassa obsoleta* embryos has long been believed to rely solely on the inheritance of determinants relayed through an early nucleate cytoplasmic protrusion, the polar lobe, to a dorsal lineage of cells (Fig. 1). Loss of the polar lobe, or the D cell that inherits the contents of the polar lobe results in a loss of cell fates along the embryonic axes. Morphological structures such as eyes, foot, statocysts, operculum, retractor muscle, intestine, heart, larval bud cells, and shell are lost (Atkinson, 1969; Clement, 1952). However, cell signaling events required for polar lobe dependent axial specification have now been identified, overturning the assumption of autonomous activation of the polar lobe determinants.

At least four signaling events are required to induce cell fate specification along the dorsal-ventral axis. First, micromeres formed at third and fourth cleavage activate the inductive abilities of the

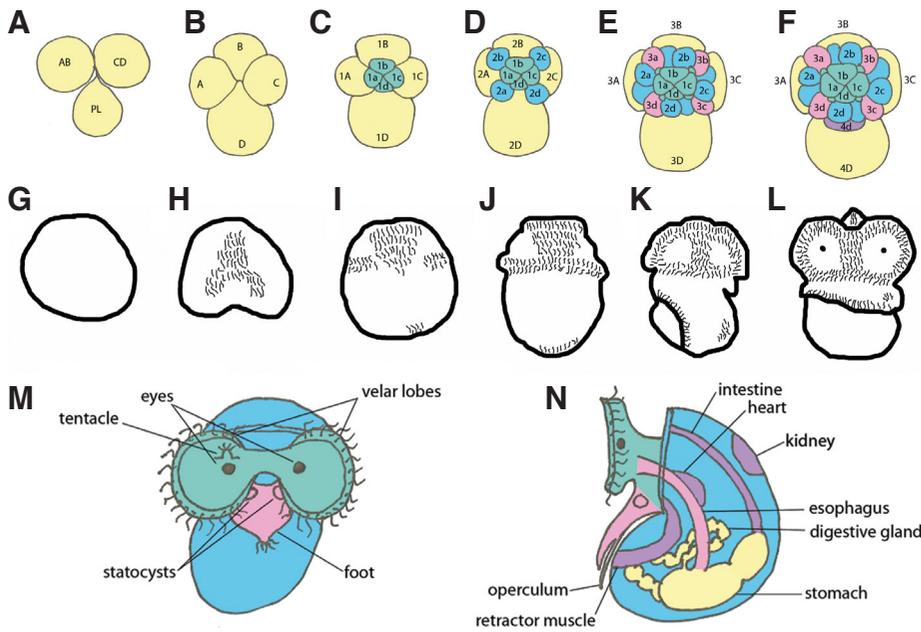
dorsal founding cell, the D macromere (Wandelt *et al.*, submitted; see Fig. 1 for nomenclature and Fig. 11 for a model of the early signaling events required for cell fate determination). Secondly, the activated D macromere (3D) induces proper cell fates along the dorsal-ventral axis in the micromeres formed after fifth cleavage (Clement, 1962; Fig. 11). Thirdly, the daughter cell of D macromere in the next cleavage cycle, 4d, completes the induction of cell fates of the other micromeres along the dorsal-ventral axis (Rabinowitz *et al.*, 2008; Fig. 11). Fourthly, the endoderm (3ABCD) maintains induced micromere cell fates for several hours after the completion of inductive signaling (Cather, 1967; Fig. 11).

Disruption of any of these four signaling events has severe consequences on axial patterning and cell fate specification (see Fig. 11). Loss of the activating micromeres prior to fifth cleavage (Wandelt *et al.*, submitted) or the D macromere prior to inductive

*Abbreviations used in this paper:* DAPT, (N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-Butyl Ester)DI, Delta; N, Notch; N-ICD, Notch's cleaved intracellular domain; SuH, Suppressor of Hairless.

\*Address correspondence to: Lisa M. Nagy, 1007 E. Lowell, Molecular and Cellular Biology, University of Arizona, Tucson, AZ 85721, USA. Tel: +1-520-626-2368. Fax: +1-520-621-3709. E-mail: lnagy@u.arizona.edu

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**Fig. 1. Developmental stages and fate map of *Ilyanassa obsoleta*.** A-F are cleavage stage embryos. (A) During first division, an anucleate sac of cytoplasm called the polar lobe (PL) is extruded. (B) During second cleavage, four macromeres (ABCD; yellow) are formed and the D cell is much larger due to exclusive inheritance of the polar lobe material. (C) The macromeres divide toward the animal pole in a synchronous manner to form much smaller cells called the first quartet micromeres (1abcd; teal). (D) The macromeres divide again to form the second quartet cells (2abcd; blue). (E) The macromeres divide again to form the third quartet cells (3abcd; pink) and the second quartet cells divide (blue). (F) The 3D macromere divides much earlier than the other third order macromeres to form its daughter micromere, 4d (purple). G-L are post-cleavage stage larvae. (G) Larva at the end of the first day of development after the completion of epiboly. (H) Larva at the end of the second day of development after gastrulation has begun. Apical cilia have begun to differentiate. (I) Larva at the end of the third day of development, organogenesis is ongoing. (J) Larva at the end of the fourth day of development. (K) Larva at the end of the fifth day of development. (L) Larva at the end of the sixth day of development after the completion of organogenesis. This is the fully developed veliger. (M,N) Veligers with generalized clonal contributions with color-coding that matches cells in Fig. 1F. Generally, first quartet cells give rise to the velum (teal), second quartet cells give rise to the shell (blue), third quartet cells give rise to the esophagus and foot (pink), 4d gives rise to the intestine, heart, kidney, and retractor muscle (purple), and 3ABC/4D give rise to the endoderm and nutritive yolk (yellow) (Render, 1991; Render, 1997).

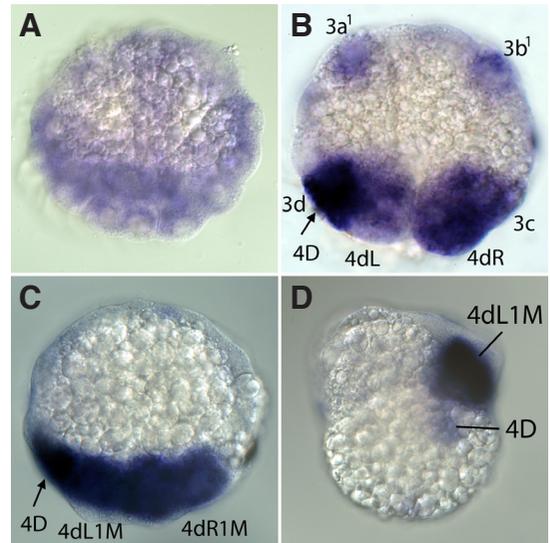
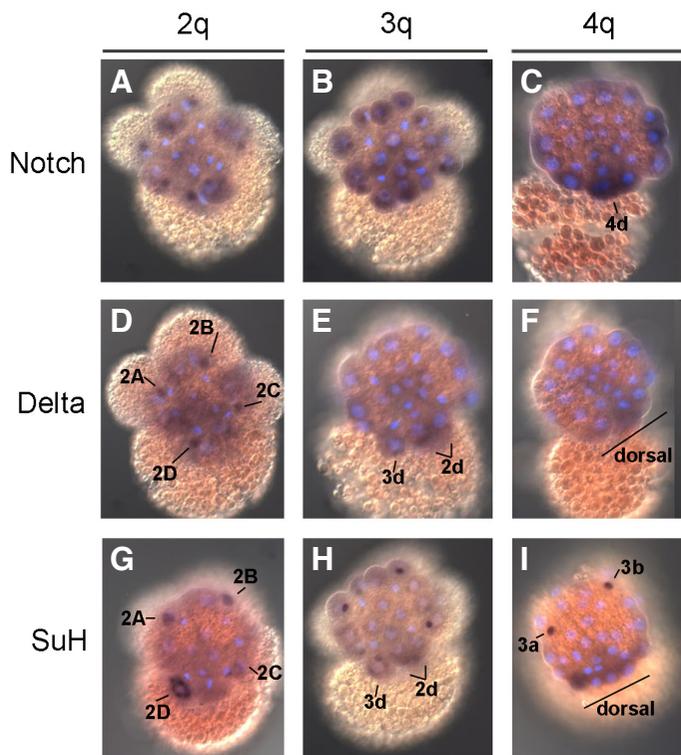
signaling during fifth cleavage, mimics the phenotypic consequences of polar lobe loss (Clement, 1962). Loss of 4d prior to the completion of its induction of micromere cell fates results in veligers with reduced velum, foot, shell and absent or disorganized endoderm as well as its direct mesendodermal derivatives (heart, intestine, and kidney; Rabinowitz *et al.*, 2008). Loss of the endoderm (3ABCD) several hours after the completion of induction results in a failure to specify cell fates along the embryonic axes in a manner that mimics polar lobe ablation, early micromere ablations or early D macromere ablations (Cather, 1967).

With few exceptions, the molecular nature of these signaling events is not well characterized. One exception is the activation of MAPK in the 3D macromere as a result of cell contact dependent activation by the micromeres. MAPK is a critical component of the signal transduced through direct cell contact between the 3D macromere and the micromeres required for proper cell fate specification in the micromeres (Lambert and Nagy, 2001; Wandelt *et al.*, submitted). Loss of the polar lobe results in a failure to properly activate MAPK in the 3D macromere and the induced micromeres (Lambert and Nagy, 2001). However, inactivated MAPK is abundant in all macromeres and the micromere signal selectively activates the inductive ability of the 3D cell and the MAPK in this macromere (Ober *et al.*, in prep.). MAPK activation in 3D is likely not sufficient to explain the inductive capacity of that cell, as embryos that fail to activate MAPK in 3D can still properly activate MAPK in the micromeres (Wandelt *et al.*, submitted). These data suggest that multiple, independent signaling cascades are involved in the induction of cell fates by 3D/4d. Thus, any candidate search for signaling pathways involved in axial patterning in *Ilyanassa* should not be restricted to signals that inhibit MAPK activation in 3D. Signals that have no effect on MAPK activation in 3D, yet still result in axial patterning defects should also be considered.

The Notch (N) signaling pathway fits several known requirements for the activation signal of the *Ilyanassa* dorsal cell lineage. N signaling requires cell-cell contact (Fiuza and Arias, 2007) and cell-cell contact is required for dorsal cell lineage activation and for induction of nearby cells by the dorsal cell lineage (Sweet, 1996; Wandelt, 2005). N signaling has also been implicated in mesendodermal specification (Sherwood and McClay, 1997; Sherwood and McClay, 2001; Sweet *et al.*, 2002). The daughter cell (4d) of the initial inducing cell (3D) both completes dorsal induction of micromere cell fates (Rabinowitz *et al.*, 2008) and gives rise to mesendodermal organs (Clement, 1986b; Render, 1997). Thus, N signaling is an excellent candidate to function in this early inductive patterning event.

N is a single pass transmembrane receptor protein and signaling is transduced subsequent to the binding of one its ligands. Canonical N ligands are also single pass transmembrane proteins and are members of the DSL (Delta/Serrate/lag-2) family of proteins. Signal transduction is achieved by the release of the intracellular portion of the N receptor via  $\gamma$ -secretase proteolysis upon ligand binding (reviewed in Lai, 2004; Fiuza and Arias, 2007). The cleaved intracellular domain (N-ICD) translocates into the nucleus and associates with a DNA binding protein CSL (for human, CBF1; *Drosophila*, Suppressor of Hairless; *C. elegans*, lag-1) and other transcriptional regulators to regulate transcription of N target genes (Bray 2006; Artavanis-Tsakonas *et al.*, 1999; Kadesch 2004). In the absence of N-ICD, CSL acts with other co-repressors to suppress N target gene transcription (Bray, 2006; Kopan and Ilagan, 2009). This canonical N pathway is conserved across species (Artavanis-Tsakonas *et al.*, 1999; Bray, 2006; Kopan and Ilagan, 2009). This explanation of the most basic properties of N signaling does not convey the complex mechanisms that regulate N-DI signaling (see recent reviews by Dominquez, 2014; Hori *et al.*, 2013). To what degree





**Fig. 3 (Left).** Expression of *loN-DI* pathway components during the early cleavage stages. **(A)** *loN*: At the 2q stage, all cells in the embryo have a low level of cytoplasmic expression. 2q cells have slightly stronger cytoplasmic expression. All cells in the embryo, with the exception of 1q cells, have centrosomal localization. **(B)** At the early 3q stage, all cells in the embryo have cytoplasmic expression. All cells with the exception of 1q cell have centrosomal localization. 2d and 3d cells have slightly stronger expression than all other cells. **(C)** After the birth of 4d, all cells in the embryo have a low level of cytoplasmic expression. 2q cells have slightly stronger diffuse expression. 3d and 4d also have strong diffuse expression.

**(D)** *loDI* expression: at the 2q stage, low level cytoplasmic staining is observed in all cells of the embryo. Abundant centrosomal localization is observed in the macromeres (2ABCD). **(E)** At the 3q stage, all cells of the embryo have a low level of cytoplasmic staining. Less abundant centrosomal staining is observed in 3abc. Strong cytoplasmic expression is observed in 2d and 3d. **(F)** After the birth of 4d, low level cytoplasmic staining is observed in all cells of the embryo with strong expression in the dorsal lineage cells. **(G)** *loSuH*: at the 2q stage, low level cytoplasmic staining is observed in all cells of the embryo. Centrosomal expression is observed in 3A and 3B macromeres. Perinuclear staining is observed in 3C and 3D macromeres with much more abundant expression in 3D. Less abundant centrosomal staining is observed in the micromeres. More abundant cytoplasmic staining is observed in 2d. **(H)** At the 3q stage, low level cytoplasmic staining is observed in all cells of the embryo. Centrosomal staining is observed in all cells of the embryo except for the macromeres and the 1q cells. Strong cytoplasmic staining is observed in 2d and 3d. **(I)** After the birth of 4d, low level cytoplasmic staining is observed in all cells of the embryo. Centrosomal staining is observed in 3a and 3b. Strong cytoplasmic staining is observed in the D lineage cells.

**Fig. 4 (Right).** Expression of *loN-DI* pathway components during early 4d lineage proliferation by RNA *in situ* hybridization. Views in A-C are of the animal pole with dorsal (D quadrant) down. **(A)** *loN* expression around the 54 cell stage, when the 4d lineage consists of 4dL and 4dR. Expression is ubiquitous, the stronger expression on the dorsal side is likely due to the higher number of cells present there because of the 4d lineage. **(B)** *loDI* After 4d divides, around the 45 cell stage, *loDI* is strongly expressed in the 4d daughters 4dL and 4dR, as well as 3c and 3d derivatives. It is more weakly expressed in the 3a' and 3b' cells, and on the centrosomes in 3A, 3B and 3C. **(C)** At the 59 cell stage, *loSuH* is expressed in the mesoteloblasts 4dL1M and 4dR1M. It is also in 4D, which underlies 3d' and 3d'', giving the appearance of expression in those cells. **(D)** Embryo at the same stage as in (C) viewed from the left side with the animal pole up and the dorsal side to the right, showing staining in 4dL&R1M cells but not in the overlying micromeres.

is also largely restricted to the 4d lineage (Fig. 4C). At the 54 cell stage, the transcript is strongly expressed in the bilaterally paired teloblast cells 4dL1M and 4dR1M (Fig. 4D). It is also abundant near the nucleus of the 4D cell. This cell only contributes to cells of unknown function within the digestive lumen and is not required for normal morphogenesis (Clement, 1962; Render, 1997). These expression patterns are all within the first 10 hours of development (see Goulding, 2009 for a precise and elegant timetable of early *Ilyanassa* development), the expression patterns of *loN/D/SuH* beyond the first day of development are not reported here.

#### Embryonic inhibition of N signaling results in a range of veliger defects

To determine the effects of DAPT on early embryonic devel-

opment, embryos were continuously incubated in 25μM DAPT from one cell to 150 minutes after the birth of the third quartet of micromeres (3q+150). This incubation period encompasses the period when 3D is induced to become the embryonic organizer, and 3D/4d induce specific cell fates in the micromeres. After this time the embryos were washed from the drug, and DAPT treated and control embryos were cultured to veliger stage (7 days, RT) and scored for defects. The most severely affected DAPT treated veligers had a striking phenotype, and in general appeared as “floating heads” that swam in circles to the right when viewed in their sea water dishes (Fig. 5F, 10D). In general, DAPT treated veligers had differentiated head tissue but significantly reduced or absent shell. In many of these veligers the internal organs were reduced to a yolk mass with no distinguishable features. A bi-lobed velum

and foot were present, but were frequently reduced in size. Eyes and statocysts were present. The esophagus was present but typically stunted. Intestines were present, but typically abnormally positioned. The distinguishing physical characteristics of other endodermally derived organs- stomach, digestive glands, and style sacs could not be conclusively scored in the small mass of internal tissue at the base of the head, but were either abnormally formed or missing. Other structures that derive from 4d – larval retractor muscle and hearts were not scored. Thus, veligers displayed a range of defects including abnormally formed endoderm and structures that depend on 3D/4d induction - reduced velum, reduced foot, small or no shell formation. Thus, inhibition of N signaling affects endoderm formation and many of the cell fates induced by 3D/4d, as well as the 4d mesendodermal lineage.

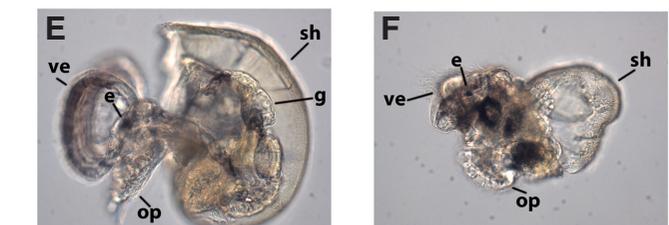
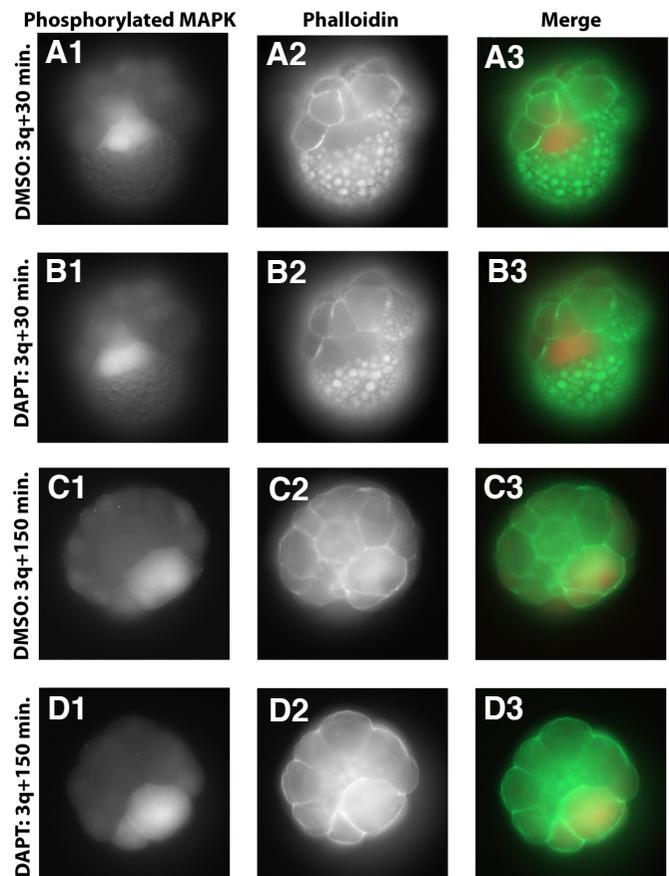
To further evaluate our results, we grouped the veligers resulting from DAPT treatment into the following four categories (Fig. 10). 1) “Normal” - This category included embryos that had a small retraction of the internal organs from the shell that is sometimes observed in normal development; 2) “Least” - veligers typically displayed *normal* shell, velum and foot development. Endodermally derived internal organ formation was severely affected. Internal organs were either missing or condensed at base of head within the shell. 20% of these veligers had minor abnormalities associated with more anterior structures, including velum, foot, eyes, operculum; 3) “Intermediate” – veligers that display *reduced* shell, velum and foot development and absent or severely disrupted internal organ formation. 93% or more of these veligers had abnormalities associated with more anterior structures; 4) “Severe” – veligers which display *reduced velum* and foot, *absent or extremely rudimentary* shell formation, and absent or severely disrupted internal organ formation. All of these veligers had varying degrees of abnormalities associated with the head.

Categorization of the resulting phenotypes was based predominantly on the effect on the shell. All affected veligers had obvious defects in endodermally derived organs, and within each category these defects ranged from the internal organs appearing either condensed or poorly separated within the shell, or missing entirely, with only yolk visible. The degree to which the most anterior structures, the velum and associated head structures, were affected was also variable within each category. In many cases the head appeared normal, but abnormalities in the head appeared with an increasing frequency as the overall severity of the phenotype increased. It is unlikely that these severely affected

larvae arise from a general toxic effects of DAPT because early markers of cell fate specification were normal (see below, Fig. 7), and embryos treated at late stages of development developed normally (see Fig. 10F).

**N signaling is not required for MAPK activation in 3D or induced micromeres**

Given the dramatic effect of DAPT treatment during the early cleavage stages, we asked whether N signaling functions in specification of the 3D organizer. To do this, we examined the pattern of MAPK activation after DAPT treatment. In untreated embryos, MAPK is activated in 3D shortly after the birth of the third quartet (3q+30 minutes) serves as a marker of 3D induction (Lambert and Nagy, 2001; Fig. 5 A1-A3). After MAPK activation in 3D, select micromeres that require induction activate MAPK



**Fig. 5. N signaling inhibition with DAPT has no consequence on MAPK activation but does have significant effects on morphogenesis. (A-D1) Antibody staining against phosphorylated MAPK. (A1) 3D MAPK activation in DMSO controls at 3q+30 minutes (side view). (B1) 3D MAPK activation in embryos treated with DAPT from one cell stage to 3q+30 minutes (side view). (C1) MAPK activation in micromeres in DMSO controls at 3q+150 minutes (top view). (D1) MAPK activation in micromeres in embryos treated with DAPT from one cell stage to 3q+150 minutes (top view). (A-D2) Phalloidin staining of embryos pictured in A-D1. (A-D3) Merged images of both MAPK activation and phalloidin staining. (E) DMSO control veliger grown to day 9 (side view). (F) DAPT treated veliger (from one cell to 3q+150 minutes) grown to day 9. (G) Table summarizing the data comparing DMSO and DAPT animals. Animals were scored during cleavage for MAPK activation and during veliger stage for phenotype. e, eye; g, gut; op, operculum; sh, shell; ve, velum.**

G	3D MAPK at 3q+30 min.	Micromere MAPK at 3q+150 min.	Normal phenotype	Least phenotype	Intermediate phenotype	Severe phenotype
DMSO	46 / 46	30 / 30	119 / 131	2 / 131	7 / 131	3 / 131
DAPT	35 / 35	25 / 25	0 / 36	0 / 36	18 / 36	18 / 36



**Fig. 6. Loss of 4d signaling mimics DAPT treatment. (A) Control veliger. (B) Veliger resulting from early (4d+20 minutes) 4d ablation displaying a reduced velum, foot, shell and absent/abnormal internal organ formation. Labeled structures include e, eye, op, operculum, sh, shell, and ve, velum.**

(3q+150 minutes) and the normal pattern of MAPK activation in the micromeres is thought to reflect micromere fate induction (Fig. 5 C1-C3). To determine whether N signaling is required for MAPK activation in 3D or the induced micromere cells, we treated embryos at the one cell stage with 25 $\mu$ M DAPT. Embryos were cultured continuously in DAPT until they were fixed at 3q+30 minutes and scored for 3D MAPK activation (Fig. 5 B1-3) or fixed at 3q+150 minutes and scored for micromere MAPK activation (Fig. 5 D1-3). Both 25 $\mu$ M DAPT treated embryos and sibling control embryos (treated with DMSO alone) displayed a normal pattern of MAPK activation in both the D macromere and the micromeres (3D: DMSO n=46 and DAPT n=35; micromeres: DMSO n=30 and DAPT n=25, Fig. 5A-D). Thus, inhibition of N signaling has no consequence on MAPK activation in the early embryo.

#### **Inhibition of N signaling closely resembles loss of 4d**

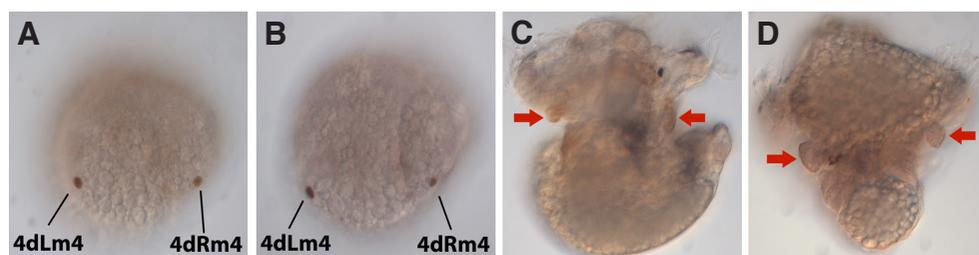
Veligers resulting from embryonic DAPT treatment and the DI siRNA display a range of defects that are generally less severe than those reported for 3D ablations (which lack both 4d inductive signaling and 4d derivatives). To better characterize the contributions of 4d inductive signaling described in a previous study (Rabinowitz *et al.*, 2008), we ablated 4d from embryos (n=37) shortly after its birth (4d+20 minutes) and scored the resulting veliger phenotypes at 7 days (Fig. 6). These animals had smaller, poorly formed velar lobes, and sometimes lacked one eye. Foot development was also poor in most larvae, and the shell was always smaller than controls. Internal structures were disorganized and yolky compared to controls and often only filled a small portion of the shell behind the head mass. These animals lacked intestines and style sacs. Clement (1986) reported that 4d deletions had stomach and digestive glands; these may have been discernable in some of our larva but were never obvious.

This may be because they were highly disorganized or because their development was delayed or obscured by yolk. Thus, the phenotypes we observe in the DAPT treated embryos resemble loss of 4d – in this case both the loss of inductive signaling from 4d (velar, foot, shell defects), as well as the loss of clonal progeny of 4d. In addition, our results confirm that 4d deletion perturbs endoderm – certainly intestine and style sac, and perhaps stomach and digestive glands as well.

#### **DAPT treatment has no consequence on early 4d specification, division, or for the morphogenesis of its clonal derivatives, the larval buds**

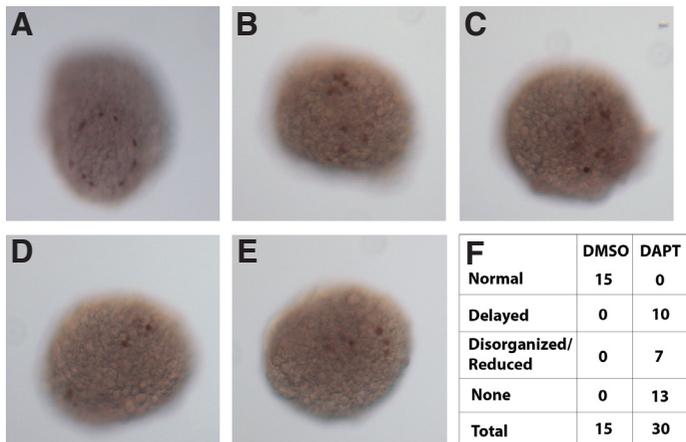
To determine whether embryonic DAPT treatment affects early 4d specification and division, we scored DAPT treated embryos for expression of the *Ilyanassa* Nanos protein that exclusively localizes to 4d descendants, as well as 4D (Rabinowitz *et al.*, 2008). Embryos were continuously treated with DAPT (from one cell to 4d+8 hours) and scored for localization of Nanos protein in 4d descendants. Normal centrosomal Nanos protein localization in two appropriately positioned descendants of 4d was observed (4dLm4 and 4dRm4, using the nomenclature of Lyons *et al.*, 2012; Chan and Lambert, in press; DMSO n=15 and DAPT n=12; Fig. 7A,B). Although the division patterns of the 4d lineage were not followed, we assume from their proper positioning in the embryo that morphogenesis had proceeded normally to that point in time.

To determine whether embryonic DAPT treatment effects late clonal descendants of 4d, we used a marker (Stat3 antibody) that exclusively identifies the larval bud cells (Gharbiah, pers. obs.). These cells are sometimes referred to as “larval kidneys” and are difficult, if not impossible, to see in the light microscope in the absence of Stat3 immunohistochemistry. They are 4d clonal derivatives (Chan and Lambert, in press). Normal bilateral larval bud development was observed in DMSO and DAPT treated embryos treated for the first 24 hours of development and scored at day 5 of development (DMSO n=30 and DAPT n=26; Fig. 7C,D). Typically, DAPT treatment during this time period (see below) results in 28% severe phenotypes, which have very poorly differentiated internal tissues. That 100% of the early veligers had larval buds detectable by Stat3 expression was somewhat surprising. These structures lie anterior to most internal organs; perhaps these larval buds develop from a 4d sub-lineage that does not depend on N signaling for normal development. These data suggest that N signaling has no consequence on early 4d specification, early 4d division, or the normal morphogenesis of at least one of the 4d clonal derivatives when scored at 5 days.



**Fig. 7. N signaling has no consequence on early 4d specification, division or morphology of its late clonal derivatives, the larval kidneys. (A,B) loNanos localization via antigen staining in embryos treated with DMSO (A) or DAPT (B) from one cell to 4d+8 hours. loNanos expression is shown localized to nucleus of 4dLm4 and 4dRm4 cells. (C,D) Stat3 antigen localization to the**

larval buds (“larval kidneys”) used as marker for 4d clonal derivative morphogenesis. (C) Normal bilateral bud formation in larvae scored on day 5 after DMSO treatment for the first 24hpt. (D) Normal bilateral bud formation in larvae scored on day 5 after DAPT treatment for the first 24 hpt.



**Fig. 8. N signaling is required for proper shell gland specification.** *Engrailed* protein exclusively localizes to the shell gland and shell gland precursor cells. (A) Four day old DMSO treated control embryo. Circular staining along the shell gland is observed with nine distinct cells staining. (B-E) Four day old embryos, DAPT treated for the first 24 hpt, displaying reduced and disorganized engrailed staining. (F) Table comparing results of engrailed staining in DMSO and DAPT treated embryos for the first 24hpt. Scored animals were categorized as either displaying temporally normal EN expression, delayed but normal EN expression, disorganized and/or reduced number of EN expressing cells, or no detectable EN expression in any cell.

**N signaling is required for proper shell gland specification**

The shell is significantly affected after DAPT treatment. To determine if inhibition of N signaling effects early shell fate specification, we used an antibody that detects the Engrailed protein (EN) and scored for proper EN localization in the shell gland of the early larva (Moshel *et al.*, 1998). Normal EN staining begins during the 2nd day of development as two arcs of cells on the right and left sides of the embryo (not shown). As development proceeds, the EN expressing cells migrate closer together to form a horseshoe shape and then enclose to form a circular pattern characteristic of the developing shell gland. By day 4, 9 distinct EN expressing cells in the shell gland are easily recognized (Fig. 8A). Embryos were treated with DAPT for the first 24 hours of development and scored for EN expression at day 4 when the shell gland is most prominent. Control veligers resulting from embryonic DMSO treatment consistently displayed a staining pattern of nine cells in the expected circular pattern along the shell gland field (n=15; Fig. 8A). Veligers resulting from embryonic DAPT treatment displayed a range of defects in EN staining (Fig. 8B-E): 33.3% of treated larvae displayed a developmentally delayed horseshoe shaped staining pattern observed in normal younger larvae (n=10), 23.3% of treated larvae display disorganized, scattered pattern of staining with fewer cells (n=7), and 43.3% of treated larvae display no staining at all (n=13). None of the DAPT veligers displayed a temporally normal staining pattern relative to controls. Thus, shell field specification, is delayed and abnormal in DAPT treated embryos.

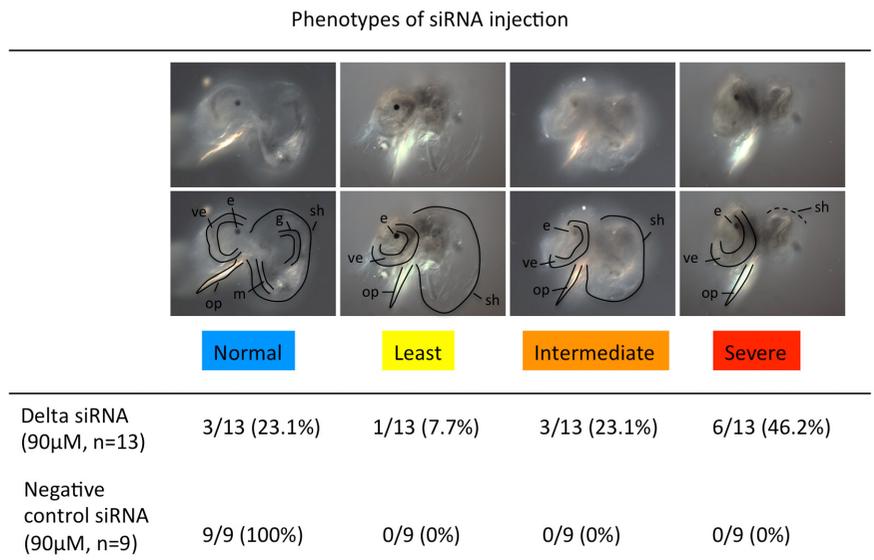
**Injection of *loDI* siRNA generates phenotypes similar to DAPT treatment**

To further test the requirement of N-DI signaling, we tested whether injection of *loDI* siRNA would mimic the DAPT treatments. 90uM *loDI* siRNA was injected at the 1-cell stage and the embryos were incubated for 7 days (Fig. 9; n=13). Injection of 20 μM *loDI* siRNA injection had no detectable effect (not shown). The veligers resulting from *loDI* siRNA had a range of phenotypes similar to those treated with DAPT (Fig. 9; n=10/13). Posterior structures such as shell, gut, intestine were disorganized or severely reduced. More anterior tissues such as the velum, eyes, foot, and operculum were normal in these embryos. The defects in more anterior structures observed in the most severely affected larvae in the DAPT treatments were not observed in these *loDI* siRNA injected veligers (compare Fig. 5F to Fig. 9). Embryos injected with a standard negative control dsRNA (Life Technologies) developed normally (n=9/9).

**N signaling is required throughout development for the proper morphogenesis of the veliger**

To determine if the phenotype observed in veligers resulting from DAPT embryonic treatment was due to inhibition of N signaling during particular developmental periods, we treated embryos/larvae at successive time points during development for 24-hour periods. A total of 28 capsules were treated; 3-5 capsules per each 24 hr treatment. Normal development of the embryo proceeds through completion of cleavage and epiboly at the end of day 1, gastrulation is primarily complete by day 2, although the blastopore is not fully closed until day 4, and organogenesis is ongoing between days 3-7 (Fig. 1; Tomlinson, 1987; Goulding, 2009; Chan and Lambert, 2014).

We found that the highest occurrence of the severe phenotype corresponds to DAPT treatment on the second day of development



**Fig. 9. Phenotypes resulting from *loDI* siRNA injections.** siRNA injected into 1 cell zygotes resulted in a range of phenotypes resembling the DAPT treated embryos. Representative larvae from each category are shown; the structures scored in these embryos are highlighted in the lower of the two panels. e, eye; g, gut; m, retractor muscle; op, operculum; sh, shell; ve, velum. In severe phenotype, the shell is partially formed (indicated with broken line). Embryos injected with control siRNA developed normally.

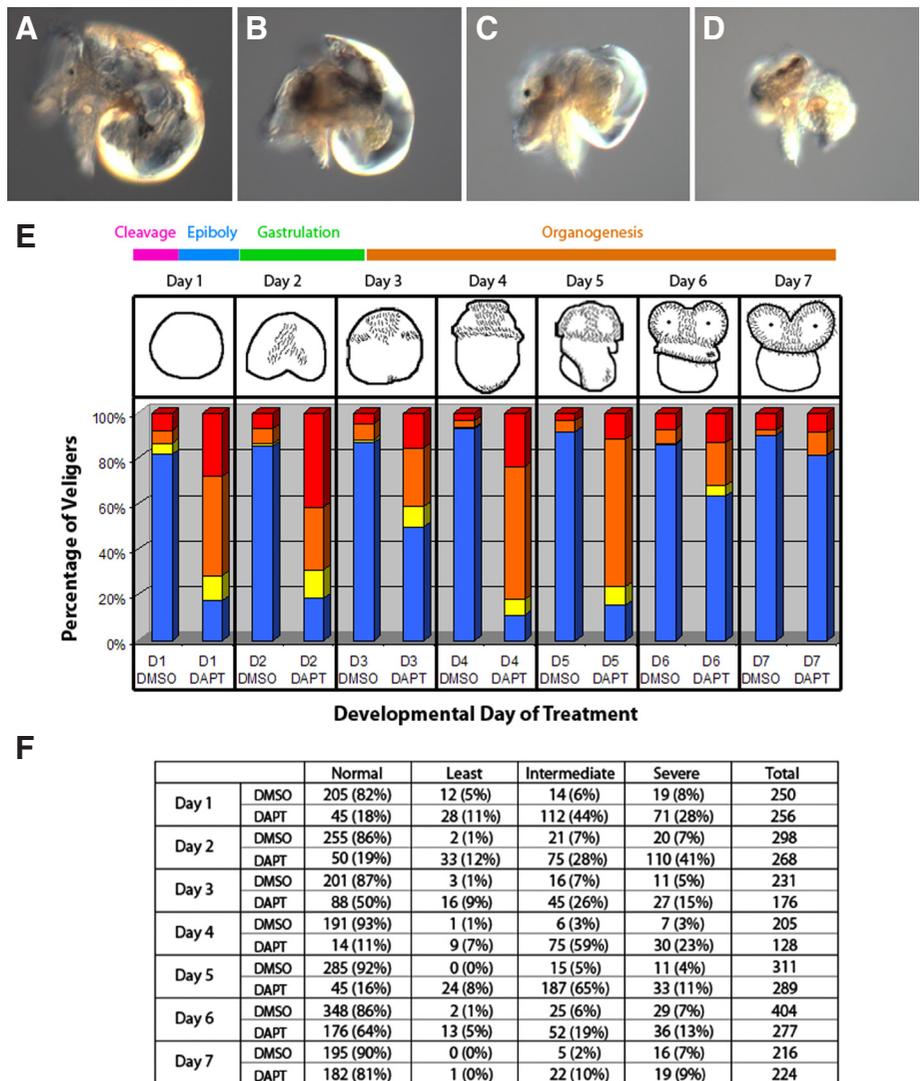
**Fig. 10. Successive 24 hour DAPT treatments throughout the development of *Ilyanassa*.** (A) Control veliger, DMSO treated. (B) DAPT treated veliger displaying a "least" phenotype: Normal shell, velum, and foot but severely affected endodermally derived internal organs. (C) DAPT treated veliger displaying an "intermediate" phenotype: Severely affected internal organs as well as reduced velum, foot, and cup-like shell. (D) DAPT treated veliger displaying a "severe" phenotype: Severely affected internal organs, reduced velum and foot, and absent shell. (E) Chart displaying successive DAPT treatment results as percentages of veligers displaying normal (blue), least (yellow), intermediate (orange), and severe (red) phenotypes. Above the chart are cartoon images of larval stages at the end of day 1-7. Cleavage and epiboly are largely complete by the end of day 1. Gastrulation is nearly complete by the end of day 2 and organogenesis is ongoing through day 7.

(41%, n=110; Fig. 10). We expect, but cannot be sure (see discussion) that this is beyond the period when 4d is required for organizer-dependent cell fates (but perhaps not for signaling from particular 4d sub-lineages). The second highest occurrence of the severe phenotypes corresponds to DAPT treatment during the first day of development (28%, n=71; Fig. 10) that overlaps with a well-established period of 3D/4d inductive signaling, and most of the teloblastic divisions of 4d that generate the sub-lineages. Additionally, we found that the highest occurrence of the intermediate phenotype corresponds to DAPT treatment on the fourth and fifth day of development (59%, n=75 and 65%, n=187; Fig. 10) during organogenesis. Treatment after five days of development results in greater than two-thirds recovery of the normal veliger phenotype (day 6: 64%, n=176 and day 7: 81%, n=182; Fig. 10). This suggests that either N signaling is required continuously through the first five days of development, or is required for sequential signaling events that have similar phenotypic results.

**Discussion**

**Expression patterns of N, DI, SuH**

Because of the complexity of mechanisms that regulate N signaling (Dominquez 2014; Hori et al., 2013), inferring function for N signaling from transcript expression patterns is risky. For example, N can receive signals from adjacent cells, or from signals on the same cell. SuH can act as a repressor of N target genes prior to N signaling, and switch to an activator on receipt of signal. In addition, there are N targets that don't depend on SuH activity. Nonetheless,



when expression patterns are correlated with functional studies, the temporal and spatial patterns of expression have the potential to direct our understanding of N-DI signaling. In particular, lack of expression in a critical lineage or timepoint could eliminate a proposed function. In the early cleavage stages, *loN* and *loDI* transcripts are ubiquitously but non-uniformly expressed, and undergo dynamic intracellular changes in expression of unknown significance (Fig. 3). Consistent with a functional role of the endoderm in signaling and/or signal reception, both *loDI* and *loSuH* are expressed in some or all of the macromeres throughout the early cleavage stages (Fig. 3). At later cleavage stages, *loN* remains ubiquitous (although not uniform) and *loDI* is expressed in the daughters of 4d, as well as on the centrosomes of the 3A, 3B and 3C macromeres (Fig. 4). *loSuH* transcripts are primarily expressed in the 4d lineage, as well as the macromere 4D (Fig. 4). Because the DAPT treatments and the *loDI* siRNA injections mimic loss of 4d, the observed *loSuH* expression patterns in particular suggest a role for N-DI signaling in development of the 4d lineage, but the expression pattern is also consistent with roles for N in 4d signaling, endoderm specification and/or endoderm signaling. A role for N and/or DI in other local signaling events that are less well characterized and potentially obscured in the phenotypes reported here is also possible.

### **DAPT treatment affects endoderm formation and cell fates induced by 3D/4d**

Veligers resulting from either embryonic DAPT treatment or DI siRNA show a range of phenotypes. The least severe phenotypes primarily show defects in endoderm. As the severity progresses, the phenotypes also include reduced and ultimately missing shell, reduced velum, reduced foot, and disorganized or missing internal organs mimicking a 4d deletion. Because MAPK expression is initially normal in all the micromeres, we propose that the initial specification of micromere fate by 3D/4d is normal, but these cells fail to maintain their fates later in development. However, it is also possible that MAPK is only indicative of one of multiple signaling pathways that induce micromere cell fate, and that micromeres require a N dependent signal from 3D/4d or that 3D/4d require a N dependent inductive signal from the micromeres.

### **Injection of *loDI* siRNA results in similar phenotypes to DAPT exposure**

Injection of *loDI* siRNA resulted in veligers with primarily posterior defects, including reduction in the size of the shell, missing endoderm and disorganized intestine (Fig. 9). Although we did not directly test for the reduction of *loDI* mRNA, with the exception of the most severe phenotype, *loDI* siRNA treatment mimicked the effects of the DAPT treatment. Given that the two independent approaches resulted in very similar outcomes, it is reasonable to consider that the *loDI* siRNA is effecting the same pathway as DAPT. The absence of the most severe phenotypes in the *loDI* siRNA could be due to a failure in the *loDI* siRNA to block *loDI* expression completely, persistent maternal protein not affected by the siRNA (consistent with the abundant early expression of *loN/DI* mRNA), alternative proteins, e.g. Serrate or other non-canonical N ligands functioning in some of the cellular interactions affected by inhibiting  $\gamma$ -secretase, or off target effects of DAPT. Delta is also known to function independently of Notch (Mok and Quinn, 2005). Nonetheless, the results suggest that shell and endoderm are the most sensitive to reduction in N-DI signaling.

### **N signaling is likely required throughout development**

Successive 24 hour treatments with DAPT revealed additional requirements for N signaling beyond the cleavage stages when 4d inductive signaling has been documented (Fig. 10). The most frequent occurrence of the severe phenotype corresponds to DAPT treatment on the second day of development (41%) followed by the first day of development (28%) (Fig. 10). Additionally, the successive treatments revealed a continued requirement for N signaling through the fifth day of development (Fig. 10). These results suggest pleiotropic functions for N signaling, similar to that observed in other metazoan animals (reviewed in Louvi and Artavanis-Tsakonas, 2012).

### **Is the 4d lineage itself disrupted by inhibition of N/DI signaling?**

Nanos expression, which marks the early 4d lineage, is normal in DAPT treated embryos, suggesting that the specification of 4d itself does not require N signaling (Fig. 7). Later differentiation of the intestine and larval buds suggest that at least some 4d derivatives also develop normally (Fig. 7). However, more severe DAPT phenotypes mimic the results of 4d ablation (Fig. 5F, 6, 10, see also Rabinowitz *et al.*, 2008). This phenotype reflects not only defects in other micromere lineages that require signaling from 4d

but could also result from a failure to differentiate structures clonally derived from 4d (Fig. 6, Fig. 7). 4d clonal derivatives might not be specified properly, or initially specified but not maintained later in development. Additional markers for the various lineages of 4d would be required to determine the degree to which the complete 4d lineage is affected in DAPT treatments.

### **N signaling is not required for MAPK activation in 3D or in induced micromeres**

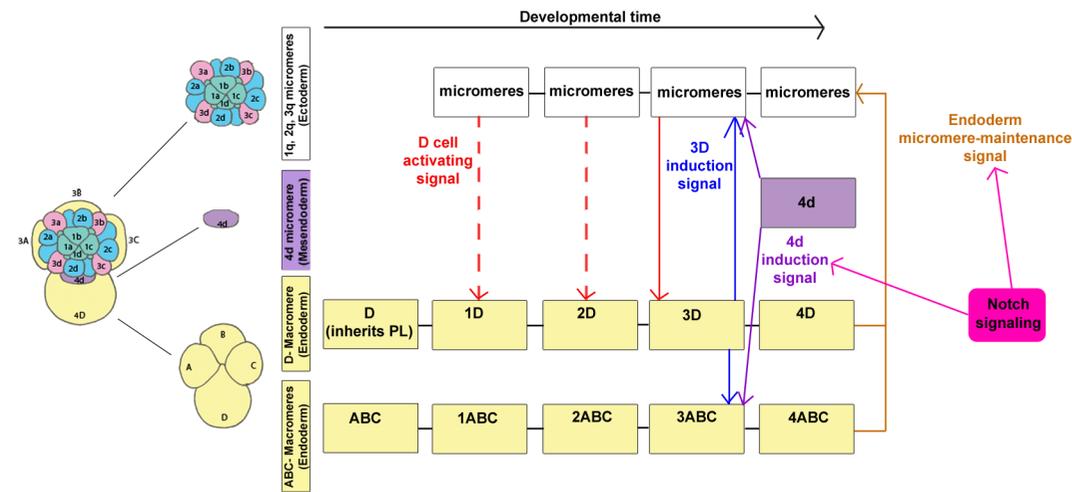
The interactions between the polar lobe containing D lineage in *Ilyanassa* and other cells in the early embryo resemble inhibitory interactions between equivalence groups regulated by N signaling in other embryos (Artavanis-Tsakonas *et al.* 1994, Campos-Ortega, 1995). Consequently, we hypothesized a role for N signaling in the activation of the dorsal lineage (3D and 4d). To test this, we used activated MAPK in 3D, which is subsequently inherited by 4d, as a marker of active dorsal lineage induction. We found that inhibition of N signaling through pharmacological drug inhibition (DAPT) had no consequence on MAPK activation in 3D or on MAPK activation in the induced micromeres. This suggests that N signaling is not required either to transduce the micromere signal to activate MAPK in 3D or to transduce the 3D/4d inductive signal to activate MAPK in the micromeres (Fig. 5).

### **N signaling is required for shell gland specification**

Larvae resulting from DAPT treatment displayed a range of EN defects including developmentally delayed but normal EN expression, scattered expression with fewer cells staining for EN, or no EN staining at all (Fig. 8). This suggests that shell gland specification either initially requires N signaling, or requires N signaling for its maintenance and proper morphogenesis. The cells in the shell field are clonal descendants of all of the 2nd quartet cells (Cather, 1967; Render, 1997; Gharbiah *et al.*, 2013; Chan and Lambert, in press) and the shell is missing in 3D deletions (Clement, 1962) and reduced in deletions of 4d (Rabinowitz *et al.*, 2008). Thus, failure to appropriately specify the shell field in the DAPT treated animals could either result from a failure to properly specify the 2nd quartet cells or a failure to maintain their specification at a later time.

### **Specification of the endoderm – is 4d involved?**

There is little in the spiralian embryology literature that directly addresses the mechanisms by which endoderm is specified. The macromeres 4A,B,C give rise to the stomach, style sacs and digestive glands (Chan and Lambert, in press), while 4D fate maps to cells in the digestive lumen (Render, 1977). 4a, 4b, and 4c contribute to the digestive glands (Chan and Lambert, in press). Clement (1962) reported that deletions of 3D/4d are missing style sacs (of 3C origin) and have severely misshapen digestive glands and stomach. The missing style sac was interpreted as lack of permissive growth conditions due to the loss of the intestine, rather than a requirement for an inductive signal from 3D/4d (Render, 1997). The 4d deletions reported here also have missing style sacs, and have more severe internal organ defects than previously reported (Clement, 1962; Rabinowitz *et al.*, 2008). In the leech and *Tubifex*, 4d or M teloblast ablation disrupts endoderm segmentation and gut differentiation (Gline *et al.*, 2011). Do *Ilyanassa* endoderm precursors require a signal from 3D/4d to differentiate as endoderm? If so, the signal is likely independent of the proposed organizer signal that activates MAPK in the micromeres, as MAPK



**Fig. 11. Model for cell fate determination in *Ilyanassa*.** The D macromere exclusively inherits the polar lobe. Micromere activation of the D cell's inductive abilities (red) is completed soon after the birth of 3D. The precise onset of this signal is not known (dashed red lines). 3D induces polar lobe dependent cell fate specification in the micromeres and other macromeres (blue). 3D gives rise to 4d and 4D after which the D macromere induction is complete. 4d continues to induce micromere cell fates (purple). The abnormal endoderm and loss of style sac from 3D/4d deletions (Clement 1967; this report) could

indicate a signal from 3D/4d to the endoderm, at least to 3C (purple). The endoderm is required to maintain induced cell fates after the completion of D lineage cell fate induction (brown) (Cather, 1967). We propose that N signaling (pink box) is involved in 3D/4d induction of cell fates (purple) and in a signaling event between the endoderm and the induced micromeres (brown). Additional functions of N are likely and are not depicted in this diagram.

expression is not detectable in any of the macromeres after the birth of 4d (Lambert and Nagy, 2001). A common defect observed in veligers resulting from DAPT treatment and DI siRNA is absent or abnormal endoderm formation. We propose that N-DI signaling could function as a lateral signal from 3D/4d to induce endodermal patterning. In sea urchins, endomesoderm is initially specified by maternal Wnt (Wikramanayake *et al.*, 2004).  $\beta$ -catenin subsequently induces the expression of DI, in the presumptive endoderm, which in turn activates the N signaling pathways in the neighboring presumptive mesoderm cells. This inhibits Wnt signaling and induces the mesodermal specification program. In the mollusc *Crepidula*,  $\beta$ -catenin is selectively stabilized in 4d sub-lineages, but knock-down blocks all differentiation in the embryo (Henry *et al.*, 2010). We find that early pharmacological stimulation of Wnt signaling with azakenpaullone results in similar "floating head" phenotypes described here for inhibiting N signaling (Harrison, Nakamoto and Nagy, pers. obs). Thus, a N-Wnt signaling interaction may share a functional role in endodermal patterning between some spiralian embryos and echinoderm embryos.

#### ***loN/DI signaling may be involved in a signaling event between the endoderm and the micromeres***

The requirement for N/DI signaling during the second day of development could be a result of defects in specification or signaling from 4d sublineages. Alternatively, (or in addition) N/DI signaling could be required to maintain 3D/4d induced fates (see Fig. 11). We suggest that our data implicates N/DI signaling in both the initial induction of endoderm and the endoderm (3ABCD) may subsequently signal via N/DI to the micromeres to maintain 3D/4d cell fate induction. A requirement for endodermal signaling in the maintenance of induced fates closely overlapping this period has been previously implicated in the *Ilyanassa* literature. Cather (1967) reported that the removal of the endoderm (3ABCD which contribute to the digestive glands, velar retractor, style sac, intestine, heart, kidney (Render, 1997)) resulted in animals that failed to differentiate 3D induced fates. Cather reported that these animals formed into hollow ball-like animals that developed cilia but did not differentiate any other discernable larval structure. While

this result in itself is not surprising, given the known role for 3D, retention of any endoderm forming cell (3A, 3B, 3C or 3D), not just the expected 3D, resulted in veligers which could achieve fairly good morphology of eyes, velum, foot, and sometimes shell which are structures known to be effected in 3D ablations. Even more surprising, removal of the single remaining endoderm precursor (in this particular study, 3C) more than 16 hours after the birth of 4d in the controls resulted in a loss of cell fate induction. These experiments resulted in animals that were hollow ball-like animals with no shell, foot, or other notable structures and were identical to the animals in which the entire endoderm was removed at once (3ABCD) and removed much earlier in development. Additionally, removal of the endoderm 40 minutes after the birth of 4d (3ABC4D) resulted in a loss of cell fate induction (Cather, 1967). Scoring the DAPT treated embryos for endoderm specific markers would further substantiate this model.

#### ***N signaling function within lophotrochozoans***

In *Ilyanassa*, the data reported above supports a role for N signaling in maintaining cell fate patterning induced by the D lineage. The only other functional report for N signaling in a lophotrochozoan is in the leech in which N signaling was implicated in segmental induction by D lineage cells (Rivera and Weisblat, 2008). If N is required to specify 4d fates, then in both species it is involved in patterning posterior teloblastic lineages. The requirement for endodermal patterning we report here is not shared with the leech, and suggests that 1) N signaling has evolved independent functions in derived annelids and derived molluscs, or 2) N signaling plays an important role in endodermal patterning and D lineage signaling in both annelids and molluscs, but the morphological consequence of this signaling has diverged with time.

#### ***Evolution of the role of N signaling within metazoans***

The discovery that N signaling is required for segmentation in annelids, arthropods and vertebrates has increased speculation regarding the evolutionary origins of segmented body plans in metazoans (Erwin and Davidson 2002; Seaver, 2003; De Robertis 2008; Couso 2009; Rivera and Weisblat, 2009, Chipman 2010; Williams

*et al.*, 2012; Erickson *et al.*, 2013). As the molluscan 4d lineage is related to teloblastic growth and by extension segmentation, a role for N signaling in the molluscan D lineage is relevant to these arguments. There are two major, competing hypotheses as to the origin of segmentation (see references above, as well as Balavoine, this issue): 1) a single origin of segmentation in metazoan animals with a single, segmented ancestral bilaterian; 2) multiple origins of segmentation in which all three clades independently evolved segmented animals. The argument for a single origin is supported by the requirement for N signaling in segmentation of representative animals from all three clades of segmented metazoans. However, within each clade of segmented animals there are numerous examples of closely related segmented and unsegmented animals. This suggests that segmentation has been lost numerous times throughout evolution or that N signaling has been independently recruited at least three times to function in segmentation. We found that N signaling in non-segmented animals such as the mollusc *Ilyanassa* has a potential role in the 4d lineage, known for its teloblastic growth – a mechanism shared with segmental growth in annelids. We also present evidence for a role for N in the function of the endodermal lineage in *Ilyanassa* – a N function shared with echinoderms – another non-segmented clade of animals. Loss of N signaling also affects the patterning of multiple cell fates including a uniquely molluscan feature, the shell. Additional sampling of other lophotrochozoans and unsegmented animals will help clarify the evolution of N signaling within the Metazoa.

## Materials and Methods

### Snail husbandry

Adult snails were obtained from the Marine Resources Center at the Marine Biological Laboratory (Woods Hole, MA) or collected from Bar Harbor, Maine or from Roberts Landing in Alameda County, California. Adult snails were maintained and embryos collected and reared as previously described (Gharbiah *et al.*, 2009). Time of development was scored as hours post-trefoil (hpt) at room temperature, the stage of early embryogenesis between first and second cleavage when the large polar lobe is fully extruded (Tomlinson, 1987).

### Cloning, orthology assignments and sequence alignments

*IoN* and *IoDI* cDNAs were cloned by polymerase chain reaction (PCR) with degenerate primers from cDNA generated from mixed stages. Primer sequences are available upon request. *IoSuH* cDNAs were cloned by PCR using gene specific primers to EST sequences (Kingsley *et al.*, 2007). *in situ* hybridizations were carried out as previously described (Lambert and Nagy, 2002). Sequences isolated by degenerate PCR were assigned orthologies based on BLASTX searches of the GenBank database at NCBI. In addition, the *Ilyanassa* EST sequences were searched for additional orthologs of N, DI and SuH. Amino acid sequences from mouse, *Drosophila*, and *Lottia* were downloaded from the protein database in GenBank. Amino acid sequence alignments were made using ClustalX, using default parameters.

### Immunohistochemistry, *in situ* hybridization

Immunohistochemistry was performed as described by Lambert and Nagy (2001) with the following alterations: Embryos used for anti-active (double phosphorylated) MAPK antibody (1:200, M8159, Sigma), anti-engrailed (1:1, 4D9, DHSB), and anti-nanos (1:50, Rabinowitz 2009), mouse anti-STAT (Chemicon MAB3705) were fixed for 30 minutes in Pipes-EGTA-Magnesium (PEM) (100 mM PIPES [pH 6.9], 10mM EGTA, and 1mM MgSO<sub>4</sub>) with 4% paraformaldehyde and 0.1% Triton X at RT.

### DAPT drug treatment

10mM DAPT (N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-Butyl Ester; Calbiochem) stock in DMSO was stored at -20°C. Working concentration of DAPT was diluted in Filtered Artificial Sea Water (FASW) just prior to use. To determine drug concentration for experiments a dose response curve was performed. At concentrations higher than 25μM more than half of the embryos did not survive to the veliger stage. At lower concentrations, an increasing percentage of the larvae developed normally. Therefore for all drug treatments embryos from the same capsule were incubated in FASW containing either DMSO (2.5%) or 25μM DAPT (2.5μL of 10mM DAPT in DMSO/mL of FASW). After drug incubation, embryos were washed three times in FASW and reared in 0.2μm-filtered artificial seawater (FASW, Instant Ocean) at RT (21°C) with penicillin (10 units/mL) and streptomycin (10 μg/mL) (7-9 days). Variation in rate of development both among siblings from the same capsule (small variation) and between capsules (much larger variation) has been noted in the literature (Tomlinson, 1987). Therefore, all 24-hour treatments that involve multiple capsule treatments have approximate start and end times of ± 6 hours for the stated period of developmental incubation.

### Injection of DI dsRNA

Double-stranded Stealth siRNA (Life Technologies) was used for the DI RNAi experiment. The following sequence (5' to 3') for DI siRNA was designed with BLOCK-IT RNAi program (Life Technologies): GACCAGTG-CAAGACCCACTTCAACT. Stealth RNAi siRNA negative control Med GC duplex (Life Technologies, catalog #12935300) was used as a control. The DI dsRNA and control dsRNA were injected at a concentration of 90μM of dsRNA and 0.5% tetramethylrhodamine (Fluoro-Ruby) 10,000 MW, lysine-fixable (Life Technologies).

### 4d deletions

4d was ablated by a glass needle roughly 20 minutes following its birth. The cell was removed and the larvae were cultured for 7 days prior to fixation.

### Scoring and fixing larvae

Injected and drug treated embryos were fixed after rearing to veliger stage and most of the internal nutritive yolk had diminished allowing for organ visibility (7-9 days). Veligers were relaxed in a mixture of two parts FASW with one part saturated trichlorobutanol, and then fixed with 3.7% formaldehyde in FASW for 30 minutes at room temperature and mounted in 80% glycerol+4% n-propyl-gallate in PBS.

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