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Expression of Axdazl and Axvh in Axolotl Germ Cells, Suggest that Regulative Germ Cells Specifications is a Primitive Trait Conserved in the Mammalian Lineage

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EXPRESSION OF AXDAZL AND AXVH IN AXOLOTL GERM CELLS, SUGGEST THAT REGULATIVE GERM CELL SPECIFICATION IS A PRIMITIVE TRAIT CONSERVED IN THE MAMMALIAN LINEAGE.

By

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A Dissertation submitted to the
Department of Biological Science
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Degree Awarded:
Spring Semester, 2006

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ACKNOWLEDGEMENTS

I would like to acknowledge and thank the members of my committee (past and present): Dan Garza, Bob Reeves, George Bates, Greg Erickson, Jim Fadool, Brian Crother, John Teem, and Cathy Levenson for giving me their input and direction during my time at FSU. More particularly, I would like to thank Hank Bass for going "above and beyond the call of duty", and looking after me over the last year and more specifically, for showing me what it means to think and write like a scientist. I am proud to call him my major professor and a good friend.

I would like to thank some of my fellow graduate students, someof the best guys I have ever had the fortune of meeting, Brian Ring, Eric Sikorski, Tommy Hock, Jason Robotham, Orion Vanderlinde, Lawrence Leclare and Steve Miller. I would also like to thank Amy and Lynnette—both of them have very special places in my heart.

My lab mates were awesome. My two little sisters-- Jen and Jess, and the rest of the guys, Ferrell, Tim, Anthea, Merriedith, and Hilda made coming into work everyday an adventure, I truly miss them all. I would also like to thank Steve Miller (again), Rani Dharajen, and Chris Bacot for putting up with my outrageous cloning, and sequencing requests, and Ken Taylor, Robin Smith, and Ellen Granger for making teaching at FSU a pleasure.

Last and certainly not least, I must thank AJ Johnson, for giving me the opportunity to become a member in his lab and to work on a project that I really love. AJ was the sergeant that I would have followed into any battle. He worked hard at FSU, and did some great science. We spent a lot time together, and in all instances, he showed me infinite patience. I learned more from AJ then he will ever know, and I am truly indebted to him. I also wanted to thank my good friend and colleague Tom Masi. Tom is gifted scientist blessed with great hands. "If there is some assay or experiment that can't be done, give it to Tom and he will work it out", this was the general sentiment not just in the Johnson lab, but in all of Unit 1. Numerous times I saw him selflessly put his work down to help others. He

is a generous and loyal friend. Tom and I have seen a lot together, and he has been like a big brother to me, and I know that I would not have finished without him.

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ABSTRACT

How germ cells are specified in animal embryos has been a mystery for decades. Unlike most developmental processes, which are highly conserved, embryos specify germ cells in very different ways. The embryos of several prominent model organisms contain germ cell determinants (germ plasm) that segregate to germ cell precursors. In other animals, including mice, germ cells form in response to regulative mechanisms during development. It has been suggested that the germ line is specified similarly in urodele amphibians. To investigate the similarity between urodeles and mice, I cloned two genes from the salamander *Ambystoma mexicanum* (axolotl) that are required for proper germ cell formation in all organisms examined to date. During development, the orthologs of *vasa* and *daz*-like genes are maternally inherited in the animal cap and equatorial regions of the oocyte and are up-regulated in PGCs of tail bud embryos before the gonad forms. *Axvh* and *Axdazl* RNA display no cytoplasmic localization in gonadal PGC's, but are expressed ubiquitously throughout the female germ cell cycle. Similarly, in mouse embryos germ cells are specified by extracellular signals; they are not autonomously specified by maternal germ cell determinants (germ plasm) and I can confirm previous experiments proposing the germ cells in axolotls arise from naive mesoderm in response to simple mesoderm inducing agents (in a mechanism similar to mice). Finally, using phylogenetic and comparative analysis, I demonstrate urodele amphibians share a common mechanism of germ cell development that is ancestral to tetrapods, and retained in the mammalian lineage and that germ plasm, as found in species such as frogs and teleosts, is the result of convergent evolution; furthering the development of the axolotl as a viable experimental system for studying germ cell formation in mammals.

CHAPTER 1

SURVEY OF GERM CELL FORMATION IN MODEL SYSTEMS

The germ line in metazoans is formed from primordial germ cells (PGCs), a small group of cells that posses unique proliferative characteristics. PGCs are the precursors of the adult gametes and they exist as totipotent cells, retaining the capacity to differentiate into any other type of cell. This property of totipotency is not found any other somatic cells, which are highly restricted in their developmental potentials. The germ line is essentially immortal, and PGCs are able to maintain totipotency even through cycles of proliferation. Somatic cells, in contrast, terminate after a fixed number of mitotic divisions and unlike the germ cells, will not contribute to the next generation. Despite the conservation of these PGC properties among metazoan species, the underlying mechanisms that specify germ cells during early embryogenesis are highly variable and usually species specific (reviewed Wylie, 1993).

The segregation from the soma of the cells that comprise germ cell lineage occurs early during development. This process occurs in a cell-autonomous fashion, in species with preformed germ lines such as *Drosophila melanogaster*, *Xenopus laevis*, and *Caenorhabditis elegans* (Illmensee *et al.,* 1976; Nieuwkoop and Sutasurya, 1979; Wolf *et al.*, 1983; Strome, 1994; Williamson and Lehmann, 1996; Ikenishi, 1998). In these organisms, the germ line is set aside from the soma by a mechanism involving the localization of specific cytoplasmic determinants that are present in the 'pole plasm' or 'germ plasm' (GP) of the egg cytoplasm. During early development, the GP becomes included in the cytoplasm of presumptive PGCs and these cells become the founder cells of the germline. In contrast, some organisms form germ cells in the absence of GP. In these species, PGCs form from naïve mesoderm in response to simple mesoderm inducing agents. This induction occurs in a few species, including the some echinoderms and some mammals (Ransick *et al.,* 1996; reviewed Matsui and Okamura, 2005).

In mouse embryos GP has never been identified (Eddy, 1974; 1975), and the presumptive germ cells are clonally related to somatic cells, such as cells of the allantois and cells of the primitive blood (Lawson and Hage, 1994). Moreover, cells that would not ordinarily become germ cells, such as the presumptive somatic cells, can be made to enter the germline upon transplantation to the appropriate locations in the embryo (Tam and Zhou, 1996). Similarly, it has been suggested that germ cells form by induction in urodele amphibians such as the salamanders and newts (Nieuwkoop and Sutasurya 1976, 1979; Hanken, 1986). This mode of germ cell specification has been widely studied in axolotl embryos. Investigations have revealed that *Ambystoma mexicanum* oocytes lack germ plasm and localized germ cell determinants and that axolotl PGCs may be induced from naïve mesoderm, possibly in response to specific growth factors (Johnson *et al*., 2001; 2003), similar to the mode of induction characterized by mouse germ cells specification (Lawson *et al,.* 1999; Ying *et al.,* 2000; Minoru *et al*., 2006). The unexpected similarity in regulative germ cell formation between mice and salamanders underscores the potential of the axolotl to be exploited as an experimentally tractable model for understanding mammalian germ line formation. Such a system may afford a powerful comparative model with great potential to yield new insights into the molecular pathways that direct regulative PGC formation.

To better understand how the germ line arises in salamanders, a vertebrate with regulative germ cell development, molecular probes that could be used as germ cell markers have been identified and characterized over the last several years. These probes include axolotl homologous of known germ cell-specific genes *vasa*, *dazl* and *oct-4* (Johnson *et al.*, 2001; Bachvarova *et al.,* 2004; Masi *et al.,* in prep). The axolotl cDNA sequences were used to query the NCBI GenBank sequence database and they all showed maximum similarity to their respective mammalian orthologs (Bachvarova *et al.,* 2004). This genetic sequence similarity, coupled with the parallels between the origin and induction and migration of the germ line between axolotls and mice suggests an evolutionary conservation of the molecular and developmental basis of germ cell specification between axolotl and mouse. These findings raise interesting questions about the similarities in germ line formation between salamanders and mammals, and may indicate certain limitations to the use of some current model systems such as *Xenopus*, a species that exhibits the preformisitic mechanism.

General Review of Germ Cell Formation

Germ cells themselves are remarkably similar among species, yet alternate methods of forming the germ line in Chordata are known to occur (reviewed by Scott, 1994). In all organisms observed, PGCs arise at extra-gonadal locations within the embryo and later migrate to the gonad. They are much larger than somatic cells and contain a cytoplasmic substance called *nuage*, an electron dense, granular-fibrillar material that appears during some stage of development (Eddy 1974,1975). The PGCs give rise to the meiotic cells and thus provide the genetic continuity between generations. The similarities are notable, however the embryonic origin, and separation from somatic tissue show great variance among even closely related taxa. Interestingly, as discussed earlier, urodeles and anurans develop PGCs in very different ways.

Germ plasm is required for PGC specification in frog embryos.

The foundation of our current knowledge regarding vertebrate germ cell formation comes from historic research of anuran embryology. Bounoure (1931) first identified germ plasm in *Rana* oocytes and in the maturing and migrating PGCs during early embryogenesis. Germ plasm was later found in a variety of anurans including the frog *Xenopus laevis* (Blackler, 1958; Eddy, 1975; Wakahara 1977; Nieuwkoop and Sutasurya 1979; Ikenishi *et al.,* 1986). In *Xenopus* oocytes, the germinal granules of the GP associate with the mitochondrial cloud in early stage oocytes and localize to the vegetal cortex during oocyte maturation (Wilding *et al*., 2001). In addition, various messenger RNAs associate with the GP, including the transcripts for *Xdazl*, *Xpat*, *Xcat-2*, and *deadsouth* (reviewed Houston and King, 2000; Kloc *et al.,* 2002). Experiments involving the destruction or transplantation either of the GP either by microdissection or UV irradiation, result in embryos devoid of PGCs, indicating that the GP, and the individual components therein, act as a determinants necessary for germ cell development in anuran embryos (Smith, 1966; Tanabe and Kotani, 1974; Zust and Dixon, 1975; Ikenishi and Kotani, 1979). During the primary stages of oocyte growth, the GP associates with the METRO region of the mitochondrial cloud (Kloc and Etkin, 1998; Kloc *et al.,* 2001) and is transported to the vegetal cortex using a microtubule dependant pathway (Heasman *et al.,* 1984; Kloc *et al.,* 2002).

Upon fertilization, the GP is distributed to the vegetal-most cells within the presumptive endoderm. The cells that inherit the GP can give rise to germ cells whereas similar types of cells that do not inherit the GP develop into somatic endoderm. In the *Xenopus* blastula, the presumptive germ cells move through the endoderm and will eventually occupy the basement of the blastocoel as shown in Figure 1(reviewed Houston and King 2000; 2002). During gastrulation, they proliferate and follow the morphogenic movements of the endoderm and eventually accumulate on the posterior portion of the endoderm (Whitington and Dixon, 1975). By the tailbud stages these cells move dorso-laterally until they reach the dorsal crest of the endoderm. From there they actively migrate through the dorsal body wall, a region that will give rise to the splacnchnic mesoderm. They then move laterally to the genital ridges and eventually inhabit the gonads (Wylie and Heaseman, 1976).

PGC form from naïve mesoderm in response to common mesoderm inducing agents

Compared to anura, there is very little known about germ cell formation in urodele. Most of the research was done by Humphrey in the late 1920's or by Nieuwkoop in the mid 20th century with relatively little new data since then. The current models suggest that the germ cell forming strategies used by urodeles and anurans are quite different. Most notably, in urodeles PGCs develop in the lateral plate mesoderm, not in the endoderm as shown in Figure 2 (Humphrey, 1925, 1929; Nieuwkoop, 1947; Smith, 1964). These mesodermal cells form in response to extracellular signals from the vegetal hemisphere. The possible existence of GP in the embryos of urodele amphibians has been discussed for many years, yet this prediction was largely untested prior to the work described in this dissertation (Nieuwkoop and Sutasurya 1979; Smith *et al.* 1983). In urodeles, PGCs and a host of other mesodermal derivatives, can be induced in experiments in which cells from the animal (pigmented) region of embryos are cultured with vegetal (unpigmented) region (Figure 3; Nieuwkoop, 1969; Boterenbrood and Nieuwkoop, 1973; Michael, 1984; Maufroid and Capuron, 1985). During gastrulation, the germ cells migrate exclusively through the mesoderm, and migrate over the ventral lip of the blastopore. From there, the germ cells are positioned in the ventral posterior mesoderm. Later during tailbud stages PGCs migrate dorsally towards the gonadal ridge and ultimately populate gonads (Ikenishi and Nieuwkoop, 1978).

PGC formation in other non-mammalian vertebrates

Germ cell formation has also been examined in a number of vertebrates, and nearly all of them exhibit the cell-autonomous mode for germ cell specification. In fish, the embryonic origin of PGCs is unclear, and there are conflicting reports as to the origin of PGCs (Niewkoop and Sutasurya, 1979; Knaut *et al.,* 2002). In the teleost model system, zebrafish, (*Danio rerio*), germ cells are formed by inheritance of GP-like material consisting of specific germ cell proteins and RNAs (Knaut *et al*., 2002). Developmental restriction occurs very early during development and by the thirty-two-cell stage, four pre-PGCs are located in the marginal blastomeres (Olsen *et al*., 1997; Braat *et al.,* 2000). During later stages of zebrafish development, the PGCs can be identified as those expressing *zvh* mRNA to a group of cells that occupy four clusters in lateral, ventral, and dorsal positions of the yolk syncitial layer (Yoon *et al.,* 1997; Braat *et al*., 1999; Knaut *et al.,* 2000, 2002). During epiboly the PGCs migrate dorsally as two clusters of cells and eventually inhabit gonads, shortly after the twelve-somite stage (Weidinger *et al*., 1999; Pelegri *et al.,* 1999). Germ cell formation in the medaka- *Oryzias latipes*, the rainbow trout- *Oncorhynchus mykiss*, and the tilapia- *Oreochromas niloticus*, are specified is a similar fashion (Yoshizaka *et al*., 2000; Kobayashi *et al*., 2000).

Like the teleost fish described earlier, chick embryos cleave meroblastically and PGCs develop from ventral cells that are located in the central region of the germinal disc which later becomes the epiblast. From there, the germ cells migrate anteriorly to the hypoblast and during gastrulation they move to the germinal crescent in the extra-embryonic endoderm (Sutasurya *et al.,* 1983). Once in anterior portion of the embryo the germ cells in *Gallus gallus* can be identified cytologically by the presence of nuage (Eddy 1974; 1975). The germ cells then travel through the developing vascular system to gonadal ridge, where they leave the vascular system and eventually enter the gonads (Nieuwkoop and Sutasurya 1979). Recently, the protein CVH, a Vasa ortholog, has been shown to be a suitable marker for PGCs throughout all stages of development (Tsunekawa *et al.,* 2000). This protein associates with the mitochondrial cloud during oogenesis, and upon fertilization it is localized to the

basal portion of the cleavage furrow. It is then asymmetrically distributed to daughter cells that will become PGCs, suggesting the germ cell lineage is maternally predetermined (Callebaut, 2005).

PGC formation in mammalian embryos.

An interesting feature of mammalian oocytes is the lack of germ plasm, indicating that a very different mechanism may characterize mammalian germ line determination than that observed for non-mammalian vertebrates (Zernicka-Goetz, 1998; Ciemerych *et al*., 2000). Major germ cell markers such as *vasa* and *nanos* are not expressed in mouse (*Mus musculus*) PGCs until colonization of the gonads (Tanaka *et al.*, 2000; Toyooka *et al.*, 2000). Mouse PGCs contain high levels of alkaline phosphatase activity which can be used to detect them after 8.5 days post coitum (dpc) but not before (Chiquoine, 1954). PGCs can be traced during development, using the alkaline phosphatase marker, to a group of cells at the base of the allantois (Ginsburg et al, 1990). It appears that these cells are not lineage restricted at this time, and can contribute to both somatic and germ lineages (Soriano and Jaenisch, 1986). Lawson and Hage (1994) injected fluorescent dyes into cell of the epiblast at 6.0 dpc and were able to trace PGCs to proximal region of the epiblast adjacent to the extraembryonic ectoderm, however no injected cell gave rise solely to germ cell population. Tam and Zhou (1996) confirmed this result using grafting experiments. These results suggest that in mice, the germ line is not predetermined by maternal factors, but rather by induction at circa 7.2 dpc, just prior to the diagnostic appearance of PGC-specific alkaline phosphatase staining. Yoshimizu *et al.* (2001) showed that PGCs could be induced as early as 5.5 dpc epiblasts when cultured with extra-embryonic ectoderm. This finding suggests that cell fates are determined by positional information within the epiblast, and the action of extra-embryonic signals. In addition, at least three bone morphogenic proteins (BMPs) are required for proper germ cell formation (Zhao *et al.,* 1998; Lawson *et al.,* 1999; Ying *et al*., 2000, 2001). The PGCs then leave the extraembryonic site, and migrate over the primitive streak. From there they move through the dorsal mesoderm to the urogenital ridges where the can be identified by expression of *vasa* (Castrillion et al, 2000).

Clear lines can be drawn between the two germ cell forming strategies observed in terrestrial vertebrates. The major amphibian orders, Anura and Urodele provide excellent

illustrations of this difference. In many instances, PGC formation in salamanders and mammals is very similar. In both taxa, PGCs are derived from presumptive mesoderm. They are not specified by the inheritance of GP, but are induced by extra-cellular signals. Finally, they migrate over the blastopore lip before they inhabit the gonads. Germ cells in frogs and most vertebrates arise very differently, much like many protostomes (Nieuwkoop and Sutasurya, 1979). Germ cells in frogs are determined by the inheritance of maternal GP, derived from the endoderm, and never migrate over the blastopore lip.

Obvious parallels exist in the way that PGCs are produced in the embryos of urodeles and mammals. These observations raise an interesting evolutionary question as to which mode of germ cell determination observed in frogs and salamanders is ancestral, the regulative mode typified by mammals and salamanders or the cell autonomous mode typified by frogs and other invertebrates. Both scenarios require the evolutionary convergence of the germ cell forming mechanism, ether frogs and invertebrates or salamanders and mammals. Addressing this question is the focus of the work described in this dissertation.

Dissertation Plan

How germ cell specification occurs remains a fundamental question in embryogenesis. The embryos of several model organisms contain germ cell determinants (germ plasm) that segregate to germ cell precursors. In other animals, including mice, germ cells form in response to regulative mechanisms involving positional information and cellular signaling. To investigate germ cell determination in urodeles, where germ plasm has never been conclusively identified, I set out to clone and characterize genes expected to play key roles in germ cell development. Towards this end, I first cloned both *Axdazl* and *Axvh* from the salamander *Ambystoma mexicanum* (axolotl). The *Axdazl* gene is homologous to *Xdazl*, a component of *Xenopus* GP found in the vegetal pole of oocytes and eggs. The *Axvh* gene is the axolotl *vasa* homolog, which is expressed in gonadal germ cells in essentially all organisms studied (reviewed Raz, 2003). This work is described in chapter 2 and chapter 3 and made major contributions to two peer reviewed publications and one in preparation (Johnson *et al*., 2001; Bachvarova *et al.,* 2004; and Drum *et al*., in prep). Detailed expression and localization studies of *Axdazl* and *Axvh* mRNA were carried out to address the question of possible maternal inheritance of these transcripts. Expression profiles using

sectioned in-situ hybridization was done in collaboration with Rosmary Bachvarova at Weill Medical College, Cornell University, New York. Embryonic injections and manipulations were done with the aid of Andrew Johnson using the facility at Florida State University. The DNA sequences were obtained from the Molecular Cloning and Sequencing Laboratory at FSU. All confocal microscopy was done with the aid of Lynnette M. Gerhold, a former graduate student in the department of Neuroscience, at FSU.

Chapter four describes our work to further develop the axolotl a viable experimental model system for germ cell formation in mammals and examine the similarities between the germ cell forming mechanisms shared between urodeles and mammals relative to terrestrial vertebrates. This work contributed to two publications (Johnson *et al.,* 2003, and Bachvarova *et al.,* 2004). I also spent two weeks in the laboratory of Brian I. Crother (Department of Biological Sciences, Southeastern Louisiana University, Hammond, LA) as part of a collaboration to carry out a phylogenetic analysis of some of the sequences we use as germ cell markers and some of the results are included in Chapter 3 and form the basis of a manuscript in preparation (Drum et al., in prep).

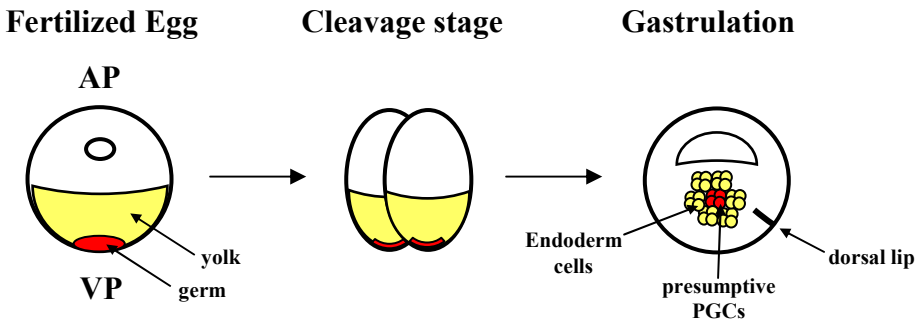


Figure 1. Diagram illustrating the origin and position of primordial germ cells in *Xenopus laevis* during early embryogenesis. *Xenopus* eggs contain germ plasm (depicted in red) along the vegetal pole, other somatic cells of the endoderm are in yellow. The germ plasm is then distributed to each blastomere during early cleavage stages. Germ plasm is then asymetrically segregated during late cleavage stages (not shown). These cells containing germ plasm are then found amongst endodermal cells during gastrulation and become primordial germ cells. Abbreviations: AP - animal pole, VP – vegetal pole.

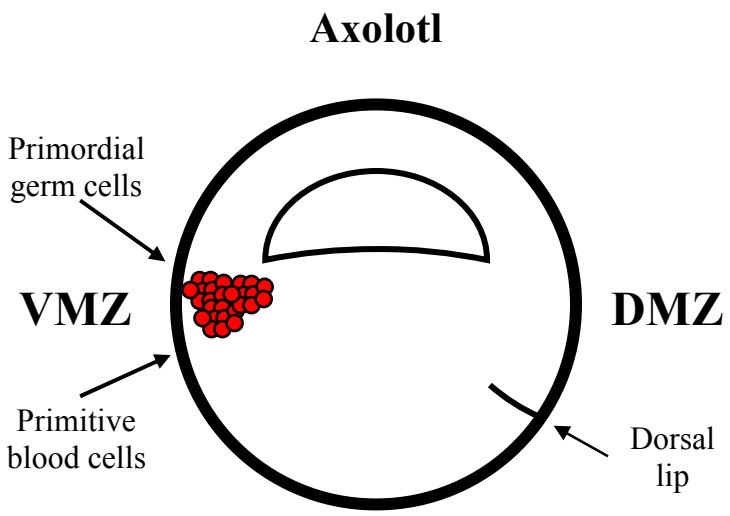


Figure 2. Lateral diagram of a gastrula stage axolotl embryo illustrating the putative position of primordial germ cells in axolotl embryos during early embryogenesis. GP has never conclusively been described in early axolotl embryos. Primordial germ cells (red) along with other mesodermal derivatives are found in the ventral marginal zone (VMZ). During gastrulation, the PGCs are the last cells to migrate over the ventral lip of the blastopore (Nieuwkoop, 1947; Smith, 1964).

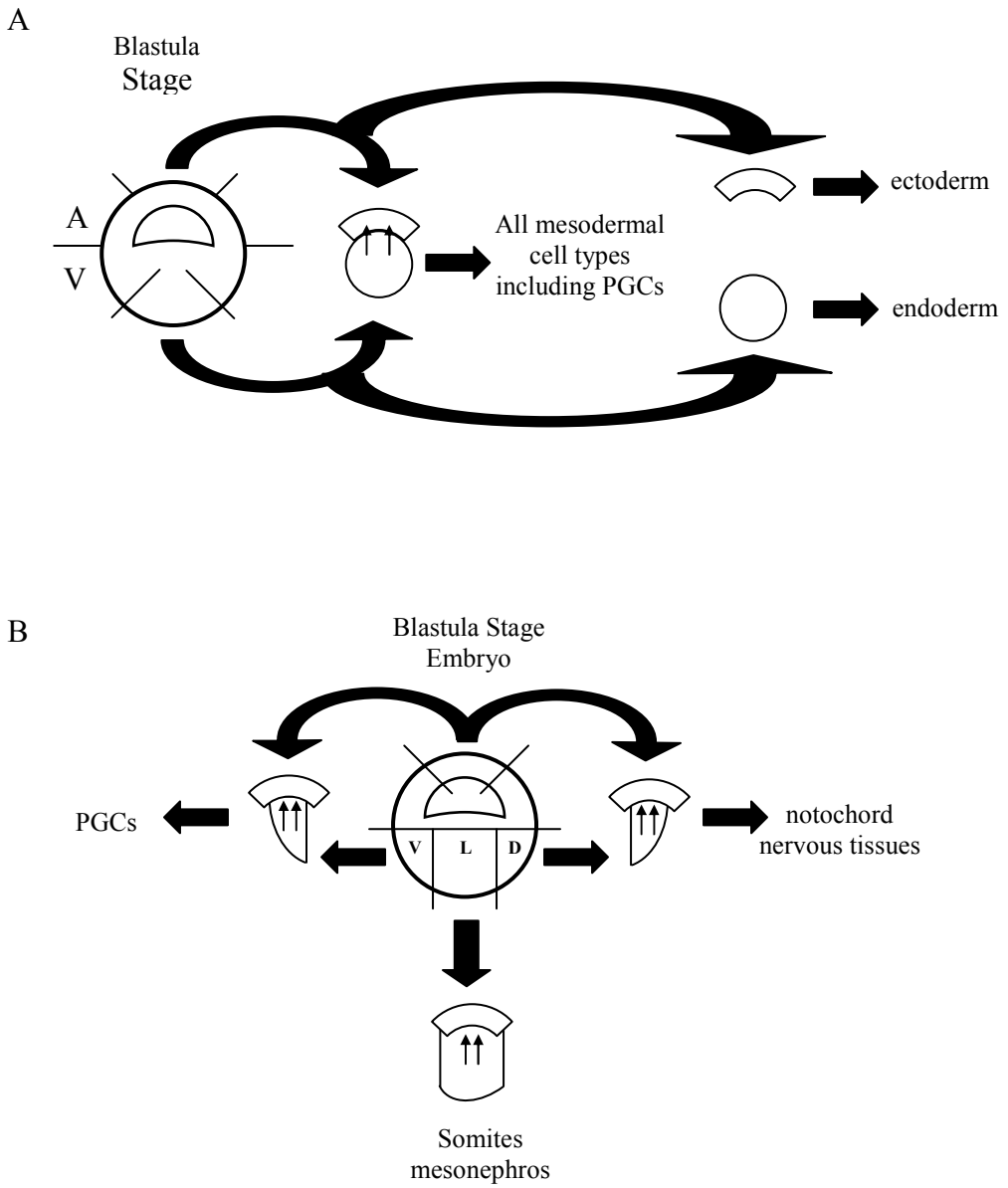


Figure 3. Illustration of recombination experiments performed by Nieuwkoop during the blastula stage of development on axolotl embryos. A) Animal caps combined with vegetal cells produce all mesodermal cell types including PGCs. When cultured alone, animal caps and vegetal cells develop into ectoderm and endoderm, respectively. B) Regional differences of the vegetal hemisphere when combined with animal caps. Dorsal regions (D) induce notochord and nervous structures. Lateral regions (L) induce dorsal mesodermal derivatives such as somites and mesonephros. Ventral regions (V) induce PGCs and other ventral mesodermal derivatives such as blood.

CHAPTER 2

CLONING AND CHARACTERIZATION OF *AXVH* AND THE EXPRESSION IN THE AXOLOTL GERM CELL LINEAGE

Background

How the germ line is segregated from the surrounding somatic tissue remains a fertile topic in animal development. Classical work performed in model organisms has revealed the presence of two widely-used strategies for germ cell formation. The germ line of *Drosophila*, *Caenorhabditis*, *Danio*, and *Xenopus* is predetermined and germ cells can be traced during development by the asymmetric distribution of *germ plasm* (GP) composed of, but not limited to, specific proteins and RNA determinants (reviewed Wylie, 1999; Houston and King 2000). In other animals, such as urchins (Ransick *et al*., 1996), and mice (reviewed Statz-Gaiano and Lehman, 2001) the germ line forms from naïve tissue in response to extracellular signals, with no evidence of an inherited GP in early embryogenesis.

There are conflicting reports as to the mechanism of germ cell formation in salamanders. Overwhelming evidence suggests PGCs in urodele amphibians are of mesodermal origin. More specifically, germ cells develop from cells located in the ventral marginal zone (VMZ) of the urodele gastrulae (Humphrey 1927, 1928, 1929; Nieuwkoop 1947, 1950; Smith 1964). It has been suggested by Smith (1964), that there may be localized germ cell determinants in the mesoderm that may alone be able to direct the formation of germ cell lineage. More recently, Nieuwkoop (1969, 1970) demonstrated that mesodermal derivatives, including germ cells, can be induced by culturing the ectodermal portion of the salamander blastula with the similar staged ventral vegetative yolk mass. This data suggests that unlike anuran amphibians, urodele PGCs arise by induction from a pool of uncommitted embryonic cells, and any part of the animal cap (ventral or dorsal) can form either germinal or somatic tissues in response to inductive cues (Sutasurya and Nieuwkoop, 1974; Michael 1984; Maufroid and Capuron, 1985). The frequency of the cell types varies however,

depending on the dorso-ventral polarity of the explants used and PGCs were only produced if ventral mesoderm was formed (Boterenbrood and Nieuwkoop, 1973). More importantly, these experiments showed that PGCs were more frequent in animal cap explants using the ventral portion of the animal cap (Sutasurya and Nieuwkoop, 1974; Michael 1984; Maufroid and Capuron, 1985). The unequal PGC forming capacity of the different regions in the urodele animal cap suggests the potential existence of determinants that increase the germ cell forming competency in the cells of this region. Similar to *Xenopus*, these determinants may be localized in newly fertilized urodele eggs, and in specific blastomeres during early cleavage stages.

In anurans, PGCs are formed from vegetal hemisphere blastomeres that retain the maternally deposited GP. While this type of GP has not been identified in urodele amphibians, comparable germ cell specific materials may be localized in urodele oocytes and later, in early stage embryos. Using histological techniques, Williams and Smith (1971), described nuage-like material resembling anuran germinal granules in the equatorial region of fertilized axolotl eggs. Electron-dense bodies have also been reported in the ventral marginal zone of blastula stage embryos (Smith, 1973). These reports suggest that the germ line in axolotl embryos may arise from specific blastomeres in the animal cap region, set aside from the soma during cleavage stages. In this scenario, much like anuran amphibians, urodele embryos could possess a predetermined mechanism for germ cell formation.

Whether urodele PGCs develop from predetermined cell populations or from unspecified mesoderm is a question that can be answered. To readdress germ cell determination in salamanders, genes that mark the germ line during development in other organisms were cloned from the salamander *Ambystoma mexicanum*. The axolotl orthologs of *Drosophila vasa* and *Xenopus Xdazl* were cloned in an attempt to determine if the expression of these genes is restricted to cell populations of the germ line.

Vasa orthologs play indispensable roles in the germ cell determination of many model sytstems

In organisms with predetermined germ lines, specific messenger RNAs can be used to mark primordial germ cells throughout development. In *Xenopus* oocytes, germ cell determining materials localize to the vegetal pole in association with aggregating

mitochondria and ribosomes to form the GP. The GP, located within the presumptive endoderm, is partly composed of various proteins and specific mRNAs associated with the germinal granules (Kloc *et al.* 2002). These GP-associated mRNAs include *Xcat2, Xpat, Xdazl*, and *DEADsouth*, all known to be required for proper germ cell formation based on transcript disruption or depletion studies (reviewed Houston and King 2000). Specific RNAs, such as *Xdazl*, and *DEADsouth* act as molecular markers for certain substages of germ cells development increasing the precision of staging for embryonic manipulation research of primordial germ cell differentiation in anuran amphibians.

Similar to *Xenopus*, germ cell formation in the teleost fish *Danio rerio* is specified by the inheritance of GP. The GP associates with the actin cortex and later a microtuble network, similar to the METRO, and late microtuble associated pathways observed in *Xenopus* oocytes (Kloc *et al.,* 1996; Kloc and Etkin 1998). From there, the aggregation of germinal granules coalesce at the distal ends of the first cleavage furrow (Braat *et al.,* 1999; Weidinger *et al.,* 1999). During cleavage stages, the GP is then limited to one side of the division plane. Daughter cells that inherit the GP will give rise to the mature germ cells. Interestingly, the mRNA *zvh* (*zebrafish vasa homolog*) is a component of the zebrafish GP, and the expression of this transcript marks germ cells from the early cleavage stages and later, throughout development (Knaut *et al.,* 2000; 2002). *Zvh* is the only mRNA characterized to date that is associated with the pre-gonadal germ line in zebrafish embryos. In contrast to other widely studied model eukaryotic organisms, where the protein product of *vasa* is a component of GP, it is the mRNA acts as a molecular marker for germ line identification and migration of zebrafish germ cells. Knaut *et al*., (2002), demonstrated that a cis-acting element in the 3' UTR of zvh targets many fish *vasa* homologs to the GP in there respective organisms. The role of this 3' UTR was shown by use of a GFP reporter gene fused to the *zvh* 3' UTR in which the reporter mRNA was localized to mitochondrial cloud in injected *Xenopus* oocytes, and GFP expression segregated with the germ cells during cleavage stages. Results from Knaut *et al.* (2002) suggest that trans-acting localization components may be evolutionarily conserved between teleosts and amphibians.

Germ cell specific RNAs in organisms with regulative germ lines is ubiquitously expressed in pre-gonadal stage embryos

Germ cells produced by regulative means are specified from naïve mesoderm during early gastrulation without the inheritance of maternal molecules. Mouse embryos provide an excellent illustration of the key features of regulative germ cell specification (Reviewed Starz-Gaiano and Lehman, 2001). In the pre-implantation stages of early mouse embryo development, GP is not detected. Later, however, as the presumptive germ cells arrive at the genital ridge, a material resembling nuage can be observed inside the cells (Eddy 1975). During the post-colonization stage, the *mvh* (*murine vasa homolog*) gene is expressed in the very cells that will give rise to the germ cell population, however *mvh* expression is limited to coincident with the onset of cellular interaction between the germ cells and the gonadal somatic cells, circa E13.5, or when the first meiotic germ cells appear in the gonads (Menke et al., 2003). Despite considerable interest, a molecular marker that associates solely with germ cells prior to urogenital-ridge colonization has not yet been identified.

Urodele amphibians may require more than germ plasm components to specify germ cells.

Ikenishi and Nieuwkoop (1978) examined axolotl embryos from a variety of stages and did not detect nuage in PGCs until colonization of the urogenital ridge, similar to observations in mouse embryos. The specification, migration and colonization of PGCs in *Xenopus laevis* has been carefully examined both morphologically, and molecularly (reviewed Houston and King 2000). It is also appears that the majority of anurans employ the germ cell forming mechanism characterized in *Xenopus* (Baliniski, 1966; Blackler, 1970; Williams and Smith, 1971). PGC determined in the other major amphibian order, urodele is limited, and primarily restricted to morphological data. A molecular marker for germ cells at any stage of development in salamanders has only recently begun to be explored (Johnson *et al.,* 2001; Johnson *et al*., 2003; Bachvarova *et al.,* 2004).

A potential candidate is the *Drosophila vasa* ortholog. The protein products of this gene are germ cell specific in the adults of all organisms observed to date (Reviewed Mochizuki et al., 2001). The gene *vasa*, initially cloned from *Drosophila melanogaster*, encodes an ATP-dependant RNA helicase and is part of a large family of proteins involved in RNA processing, including nuclear and cytoplasmic splicing, RNA editing, translation

initiation, and mRNA degradation. The diverse family consists of many proteins containing eight conserved domains in a core region of between 300 and 400 amino acid residues (Luking *et al*., 1998). *Vasa* is a member of the DEAD (Asp-Glu-Ala-Asp) box family of putative helicases similar to eukaryotic translation initiation factor eIF-4α (Schmid and Linder, 1992). *Vasa* mRNA is present throughout the *Drosophila* oocyte including the pole plasm. The protein product has also been shown to associate with nuage structure in the pole cells and is required for proper pole plasm assembly. *Drosophila vasa* been detected throughout the life cycle in the germ line, and zygotic expression is restricted to the germ cell lineage (Lasko and Ashburner, 1988; Hay *et al.,* 1988; Liang *et al.,* 1994). *Vasa*-like homologs have also been cloned in many other invertebrates such as acidians, Hydras and many insects (Nakao, 1999; Fujimura and Takamura, 2000; Mochizuki et al., 2001; Chang *et al.*, 2002).

The universal occurrence of *vasa* orthologs and their expression in the germline of vertebrates has also been documented in frogs, mice, rats, chickens, and in various teleosts (Komiya *et al*., 1994; Fujiwara et al., 1994; Komiya and Tanigawa, 1995; Yoon *et al.,* 1997; Olsen *et al*., 1997; Tsunekawa *et al*., 2000; Kobayashi *et al.*, 2000; Shinomiya et al., 2000; Yoshizaki *et al*., 2000). All of these *vasa* mRNA homologs are germline specific in adults, and in *Danio* (Knaut et al., 2000) the PGC localization of *vasa*-related mRNA in embryos has also been reported. No such data exists for salamanders until recently. We cloned the axolotl *vasa* homolog, *Axvh,* and revealed its potential as a molecular marker for germ cells in larval stage axolotl embryos as well as adult organisms, providing a much needed device for understanding how the germ line may arise in urodele amphibians.

Results

Molecular cloning axolotl Axvh

PCR using degenerate oligodeoxynucleotide primers directed towards specific *vasa*-like sequences present in *Xenopus XVLG1*, Mouse *mvh*, and Zebrafish *zvh* were used to amplify potential *vasa*-like DEAD-box cDNAs from the salamander, *Ambystoma mexicanum* (Komiya *et al.,* 1994; Fujiwara *et al.,* 1994; Yoon *et al.,* 1997; Olsen et al., 1997). Touchdown PCR (Roux and Hecker, 1997) using optimized conditions and cDNA reverse transcribed from total RNA isolated from adult axolotl testes, yielded two independent *vasa*-

like clones both approximately 400 bp. The two fragments were cloned, sequenced, and used to query GenBank DNA sequence databases with BLAST searches. We found that these two DNA fragments represented two different DEAD-box genes, one was 382 bp in length and it represents a putative *vasa* homolog similar to mouse *mvh*, and *Xenopus XVLG1*. The other clone was 402 bp in length, and it represents a MACAQT-DEAD-motif clone similar to mouse *PL-10* and *Xenopus an3* (Leroy *et al.,* 1989; Gururajan *et al.,* 1991). The *Axvh* cDNA was used to screen a bacteriophage lambda gt11 axolotl larval cDNA library at high stringency (Busse and Seguin, 1993). Three different clones from the same gene were characterized, and the largest of these cDNAs was 1223 bp in length. It consisted of a partial ORF followed by a 226 bp 3'UTR and a poly-A tail of 19 adenosine nucleotides. The 5' end including the start codon was cloned using a 5' RACE kit as described in chapter 5. A consensus full-length cDNA was determined and submitted to GenBank (accession no. AY542375). The cloned *Axvh* cDNA exhibits the presence of eight domains that typify *vasa* homologs from other species. These conserved domains include the DEAD, MACAQT, and VHRIG motifs (Linder *et al.,* 1989). The deduced amino acid sequence also showed marked similarity with mouse *mvh*, *Xenopus XVLG1*, and *DEADsouth* transcripts among other *vasa*-related DEAD box helicases (Bachvarova et al., 2004).

Axvh RNA is widely expressed in the embryo but specific to the adult gonads.

Vasa orthologs show germ cell-specific expression in metazoans (Mochiuzuki *et al.,* 2001). In many instances, such as *vasa* in *Drosophila*, and *glh-1, glh-2* and *glh-4* in *C. elegans,* the mRNA is involved in either the formation or maintenance of the germ line. To examine the spatial expression profile of the *Axvh* transcript in axolotls, RT-PCR (reverse transcriptase polymerase chain reaction) was performed using tissue specific cDNA from adult organs. *Axvh* is found in adult testis and ovaries, but not in any other somatic tissue tested see figure 4. This result suggests that *Axvh* is expressed specifically in the male and female gonads, similar to *vasa* orthologs in other vertebrates.

Xenopus XVLG, DEADsouth and zebrafish *Zvh* RNAs are maternally deposited transcripts that persist throughout development. Mouse *mvh*, alternatively, is not inherited but rather shows zygotically controlled expression later in development when the presumptive germ cells colonize the gonadal ridge. Examination of the temporal expression

of *Axvh* in axolotl embryos therefore could shed light on how the germ line develops. To examine *Axvh* mRNA inheritance, RNA gel blots containing RNA from early embryos were hybridized with a radio-labeled *Axvh* cDNA probe, yet no hybridizing sequences were visualized. Failure to detect *Axvh* RNA on gel blots could be due to the low levels of the transcript in the embryo. To overcome this limitation, RT-PCR was employed to look for *Axvh* mRNA in cDNA made from mRNA extracted from embryos during specific stages of development. Developmental staging was done according to Bordzilovskaya *et al*. (1989). *Axvh* expression was detected uniformly throughout development, and the expression remains constant through the mid-blastula transition (MBT) and during gastrula stages (stage 10-12). *Axvh* expression begins to decrease during early larval stages (stage 40) as shown in Figure 5. Expression prior to the mid-blastula transition suggests that the *Axvh* mRNA is maternally deposited even though this assay does not resolve the spatial distribution of *Axvh* within the embryo.

In an attempt to reveal the embryonic spatial and temporal expression of *Axvh*, whole-mount *in-situ* hybridization assays were carried out on early tailbud and larval stage axolotl embryos. The results were largely negative, possibly reflecting the low levels of the transcript in the embryo. We elected, therefore, to deduce the spatial expression patterns of *Axvh* from RT-PCR using carefully dissected and staged materials as detailed in chapter 5. RNA from staged and dissected embryos and reverse transcribed, and expression of *AxEF-1*α was used to standardize the amounts of input cDNA. As shown in Figure 6, *Axvh* was found to be expressed throughout the axolotl gastrula with increased expression in the endoderm and dorsal portion of embryo. During stage 34/35 *Axvh* expression remained widespread throughout the embryo with a noticeable increase in the dorsal and tail section, regions that have shown morphologically to be PGC abundant (Nieuwkoop, 1947). In stage 40 embryos, *vasa* mRNA was no longer ubiquitous throughout the embryo, but rather appeared to be dramatically reduced in the anterior regions of the head and the anterior ventral portion of the trunk, regions that were *Axvh*-rich in tailbud stage embryos. None the less, *Axvh* expression remained high in the posterior and dorsal sections.

By stage 40, feeding larval stage, *Axvh* mRNA expression was limited to a small division of the posterior trunk region where the newly developed gonads appear. Sectioned whole-mount *in-situ* hybridization has revealed that *vasa* mRNA is found in pre-gonadal

stage 38, PGCs before the gonad is formed and expression is abundant in the newly formed gonads of stage 43-44 embryos as shown in Figure 7. After differentiation of the gonad occurs, *Axvh* mRNA persisted in the gonial stage of females as shown in Figure 8. In early meiotic prophase, most of the *Axvh* mRNA was detected as concentrated nuclear spots, possibly resulting from active transcription in lampbrush loops (Callan, 1966). Finally, *Axvh* mRNA was found to exhibit a distributed pattern in the cytoplasm of late meiotic prophase oocytes, lacking any assymetrical or concentrated localized appearance as would typify a germ plasm-type localization pattern (Fig. 8, panel Q).

Discussion

We cloned the axolotl vasa ortholog *Axvh*, and found that it was expressed throughout the life cycle of gonadal germ cells. Maternal gene products of *vasa* orthologs are associated with the GP in many organisms, specifically in *Xenopus* embryos *XVLG1* plays an essential role during early migration of PGCs to the genital ridge. In addition, *Zvh* RNA the zebrafish ortholog is a molecular marker for germ cells in early stage zebrafish embryos and marks the germ cells throughout development. In mice, *Mvh* is first expressed or is greatly up-regulated as the germ cells enter the gonads, possibly in response to signals from gonadal cells. Expression of the *vasa* homolog in axolotl shares the mouse profile. In axolotl, *Axvh* expression is not restricted to the germ cell lineage until early tailbud stages, and displays ubiquitous expression in the embryo until the PGCs are in close proximity to the gonads. Nonspecific cellular expression in the pre-gastrulating embryos suggests germ cells are not specified during early embryogenesis by the inheritance of *Axvh* mRNA, as seems to be the case in zebrafish. Up-regulation of *Axvh* mRNA is at a level detectable by *in-situ* hybridization (ISH) in the early larva at stage 38, but it is not clear whether this is in response to signals from gonadal cells. Ikenishi and Nieuwkoop (1978) described the cytologically distinctive gonadal germ cells first appear in the larva at approximately stage 43, or when the nuage-rich germ cells enter the gonads, result described here confirm Ikenishi and Niewkoops 1978 observation, as *Axvh* shows cell specific expression in gonadal germ cells. However, results presented here suggest that specification may occur earlier during migration through the dorsal lateral plate. Perhaps in axolotl embryos, there is a functional maturation

of the gonadal precursor cells around stage 40, which induces a response in the germ cells similar to that seen in mouse, i.e., up-regulation of *vasa* prior to gonadal colonization.

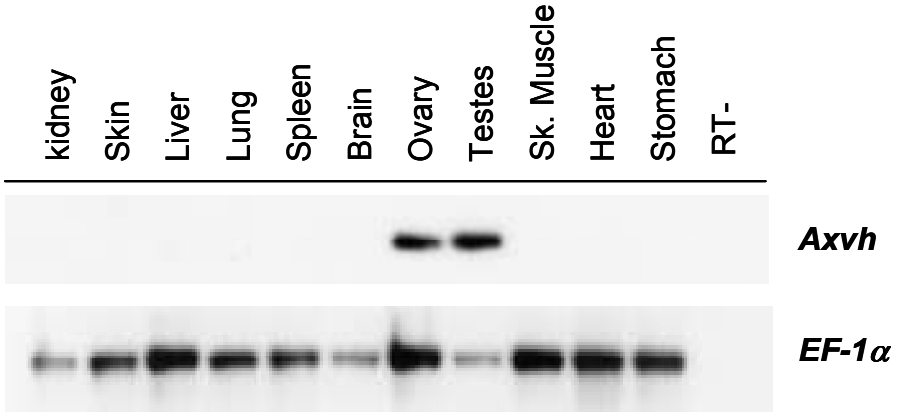


Figure 4. *Axvh* RNA is expressed only in gonadally derived tissue in adults. Equal amounts of total RNA (5µg) from the indicated tissues of an adult axolotl were reverse transcribed and used as a template to amplify either *axvh* or *EF-1α* sequences. RT-PCR was performed under identical conditions, within the linear range of amplification. Products were southern blotted and probed as described in Methods portion of chapter 5. *Axvh* RNA is detected only in cDNAs from testis and ovary. *EF-1α*, a widely expressed RNA was used as a control and shows ubiquitous distribution of expression, Sk- Skeletal.

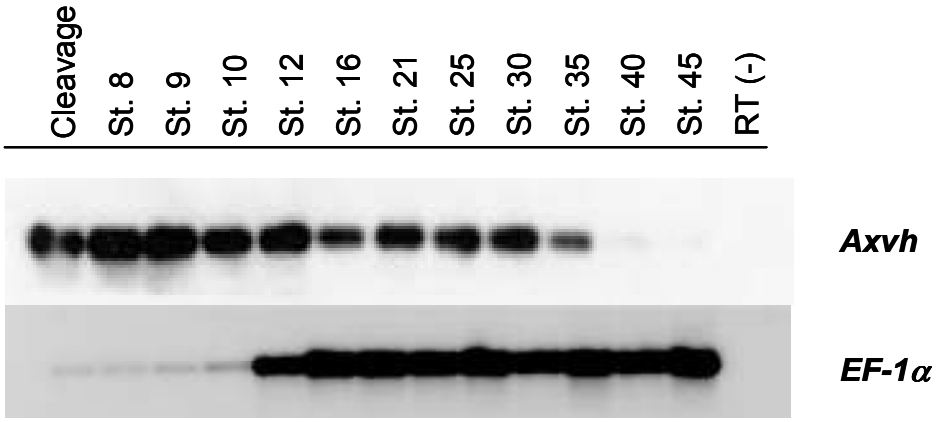
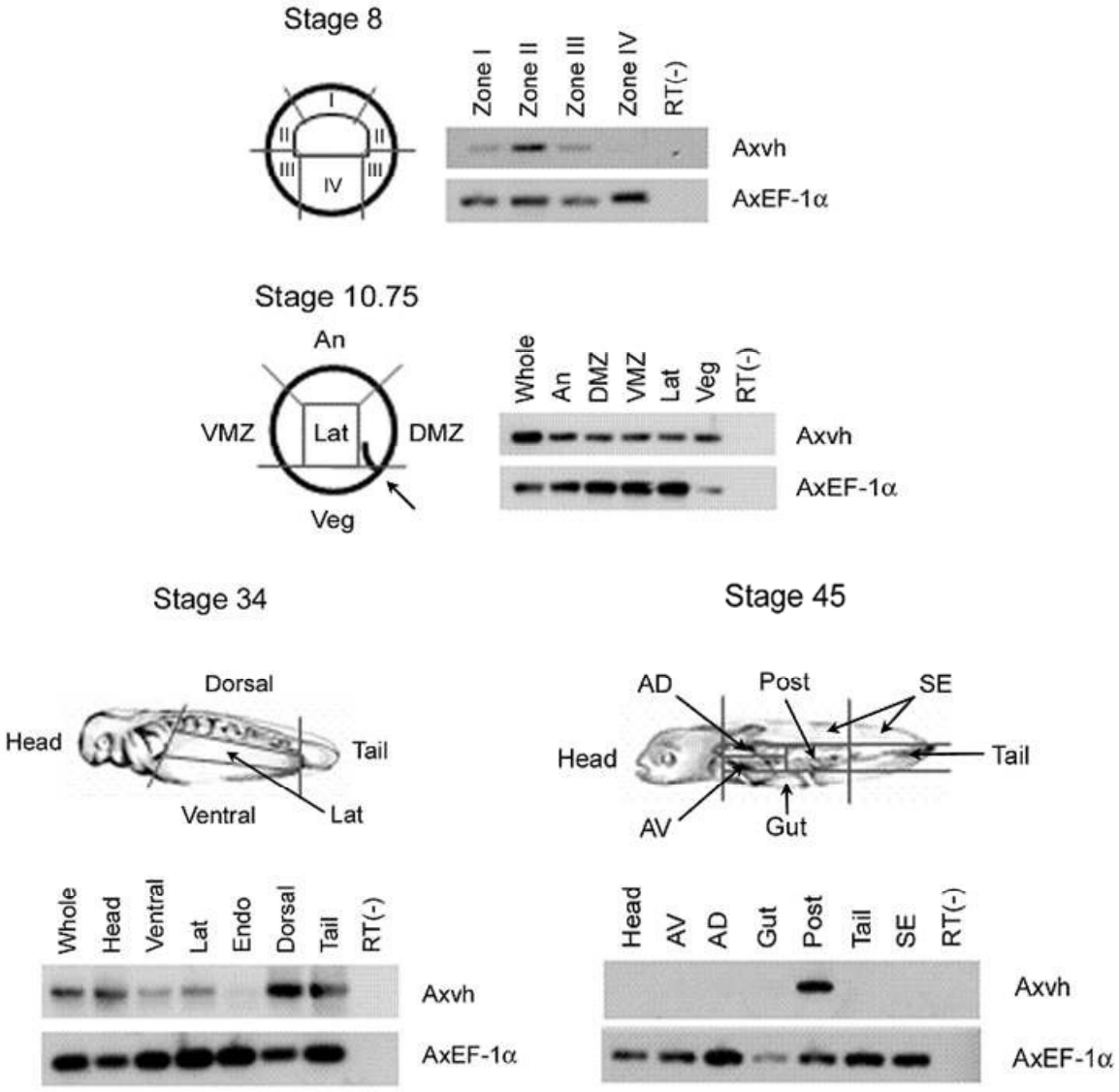


Figure 5. *Axvh* RNA is maternally inherited and is expressed throughout embryogenesis. RNA was extracted from groups of five embryos at each of the indicated stages. An equivalent amount of RNA from each stage was reverse transcribed and used in PCR to amplify *axvh* and *EF-1α* sequences. Products were detected by hybridization of radio-labled cloned sequences to southern blots as described in Materials and Methods section of chapter 2. Inherited *axvh* RNA levels remain high until larval stages (stage 40 and 45) of development when the amount of RNA drastically decreases. *EF-1α*, which becomes transcriptionally active at the midblastula transition (Stage 9, 10) is included as a control.

Figure 6. Spatial and temporal expression of Axvh in axolotl embryos. A. Stage 8 embryos. Regions I,II,III, and IV corresponding to the animal pole region, the peripheral region of the animal cap including the animal portion of the marginal zone, vegetal region of the marginal zone, or vegetal pole, respectively, were prepared as described by Nieuwkoop (1969). B. Stage 10.75 embryos. Fractions are as follows: Whole (whole embryo); An (animal pole); DMZ (dorsal marginal zone); VMZ (ventral marginal zone); Lat (lateral marginal zone); Veg (vegetal pole). C. Stage 34 embryos. Fractions are as follows: Whole (whole embryo); Head; Ventral; Lat (Lateral); Endo (Gut Endoderm); Dorsal; Tail. D. Stage 45 embryos. Fractions are as follows: Head; AV (anterior ventral mesoderm); AD (anterior dorsal mesoderm); Gut (gut tube); Post (posterior ventral mesoderm); Tail; SE (surface ectoderm). An RT (-) lane showing the product of a reaction performed with RNA from a whole embryo that was not reverse transcribed is included for each stage as a negative control . *Axdazl* RNA expression is widespread in early embryos, but is specific to the posterior ventral mesoderm, which includes the gonads, by stage 45.


Stage 8
I
II
II
III
III
IV
Zone I
Zone II
Zone III
Zone IV
RT(-)
Axvh
AxEF-1α
Stage 10.75
An
VMZ
Lat
DMZ
Veg
Whole
An
DMZ
VMZ
Lat
Veg
RT(-)
Axvh
AxEF-1α
Stage 34
Dorsal
Head
Tail
Ventral
Lat
Whole
Head
Ventral
Lat
Endo
Dorsal
Tail
RT(-)
Axvh
AxEF-1α
Stage 45
AD
Post
SE
Head
Tail
AV
Gut
Head
AV
AD
Gut
Post
Tail
SE
RT(-)
Axvh
AxEF-1α

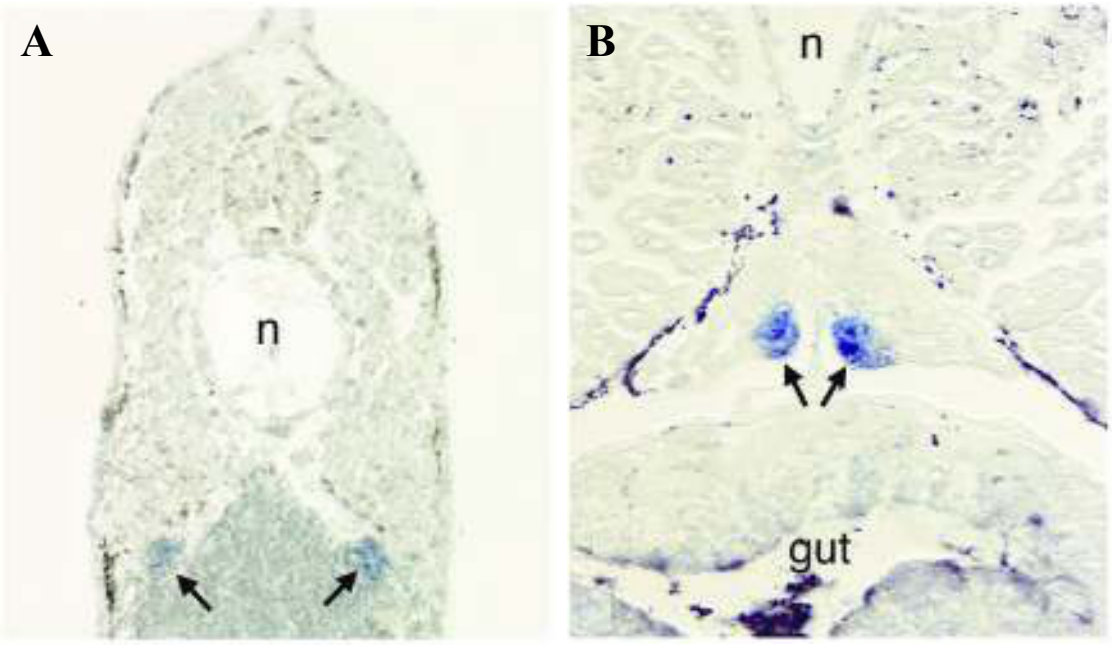


Figure 7. Expression of *Axvh* in axolotl embryos, cross sections of the posterior region. A. The earliest detection of *Axvh* expression in germ cells is stage 38. Arrows mark the germ cells migrating through the posterior mesoderm. B. *Axvh* expression in the forming gonads of a stage 45 embryo (feeding stage larvae). n, notochord.

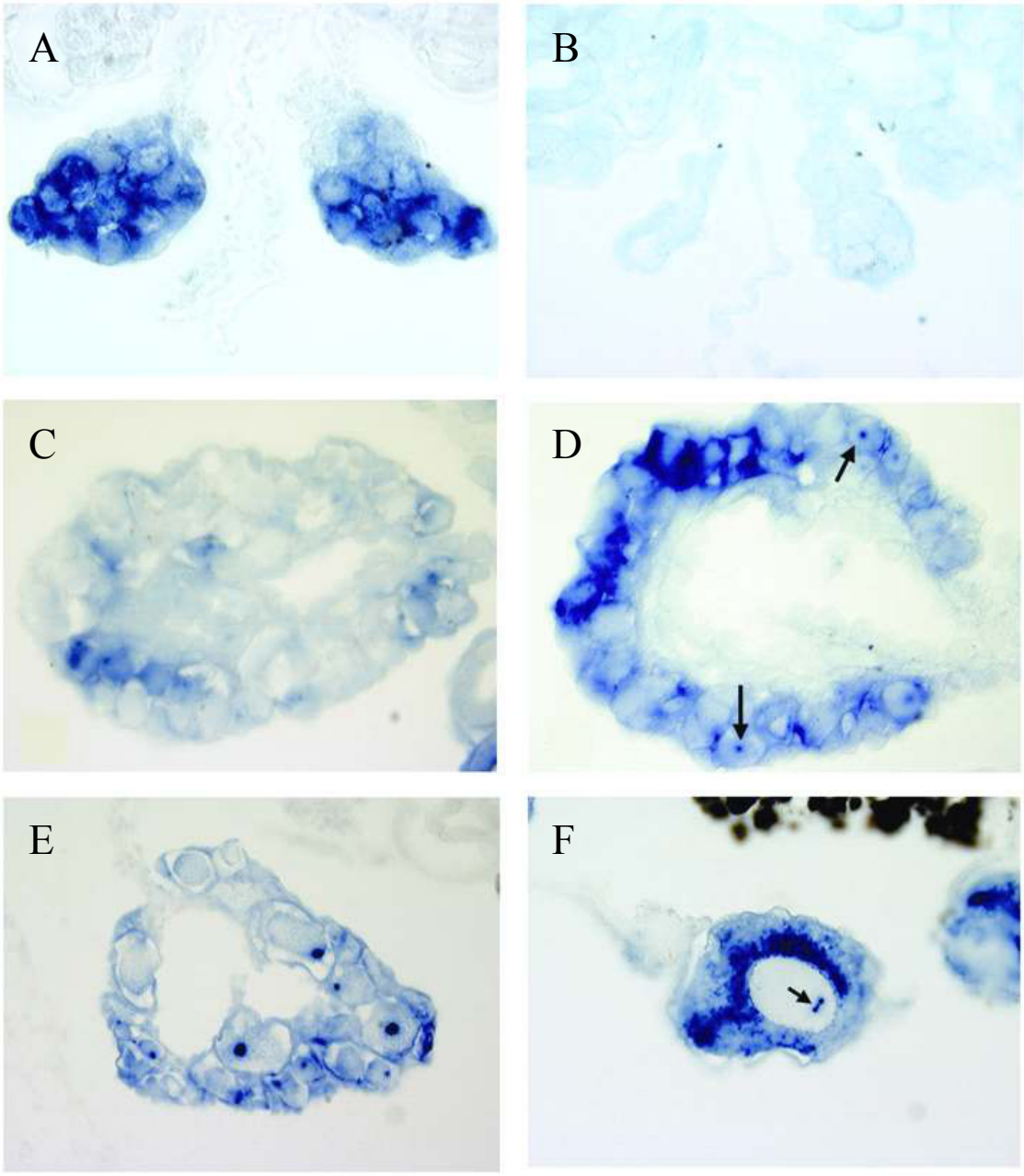


Figure 8. Expression of *Axvh* in gonadal germ cells. A. *Axvh* is expressed throughout the gonads in a stage 45, feeding stage larvae. B and C. Expression of *Axoct-4*, which is absent from stage 45 PGCs (B) as well as leptotene-pachytene oocytes [(C)(Bachvarova 2003)] was used as a negative control. D. *Axvh* expression in Leptotene-pachytene oocytes. Later oocytes have a distinct nuclear spot. E. Early diplotene oocytes with a large pale nucleus; three oocytes show a prominent nuclear spot. E. *Axvh* expression in a small growing oocyte from adult ovary with two adjacent spots in the nucleus.

CHAPTER 3

EXPRESSION OF *AXDAZL* RNA IS WIDESPREAD IN AXOLOTL OOCYTES AND EARLY STAGE EMBRYOS BUT GERM CELL SPECIFIC AS GERM CELLS APPROACH THE GONAD

Background

The *DAZ-like* genes encode an RNA binding protein and members of the gene family have been cloned from *C. elegans*, *Drosophila*, zebrafish, *Xenopus*, mouse and humans (Eberhart *et al.,* 1996; Cooke *et al.,* 1996; Saxena *et al.,* 1996; Houston *et al*., 1998; Maegawa *et al.*, 1999; Karashima *et al*., 2000). These animals include species known to utilize either of the two major germ cell specification modalities, predetermined or regulative. Gene knockout and functional disruption experiments demonstrate that the *DAZ-like* genes are essential for meiosis or gametogenesis (Eberhart *et al.,* 1996; Ruggiu *et al.,* 1997; Karashima *et al.,* 2000). In *Xenopus*, the *Xdazl* protein product is required for early germ cell development during migration toward the gonad (Houston and King, 2000). More significantly, mRNA from the *Xenopus DAZ-like* gene, *Xdazl*, is a component of the GP at all stages of oogenesis and embryogenesis providing a useful molecular marker for GP during development (Houston *et al.,* 1998).

Subcellular localization of *Xdazl* in the *Xenopus* oocyte has been well documented. During previtellogenesis, maternal *Xdazl* mRNA associates with the developing mitochondrial cloud (Zhou and King, 1996). *Xdazl* RNA is uniformly distributed in prestage I oocytes before becoming associated with the mitochondrial cloud on one side of the oocyte during previtellogenesis, stage I oocytes (Dumont, 1972). During vitellogenesis, the mitochondrial cloud shows a wedge shaped distribution extending from the germinal vesicle to the vegetal cortex, before becoming completely localized to the vegetal cortex by stage IV (Houston *et al*., 1998; King *et al*., 1999). In the developing frog embryo, *Xdazl* is abundantly expressed in the endodermal cells inheriting GP and remains associated with the GP until neurula stages (Houston *et al.,* 1998). Depletion of *Xdazl* RNA with anitsense oligodeoxynucleotides results in tadpoles deficient in PGCs (Houston and King, 2000). It

has also been suggested that *Xdazl* is required to maintain the migrational competence of PGCs during formation of the initial PGC population (Chang *et al.,* 2005).

The family of DAZ-like genes encodes RNA binding protein, leading to the idea that Dazl may play a role in translational regulation of the cytoskeletal proteins required for migration (Zhou and King, 2004). It has also been suggested that *Dazl* may act to protect the pluripotent state of the PGC's (Dixon, 1994; Houston and King 2000). Recent data shows however that embryos depleted of *Xdazl* still express the PGC-specific marker *Xpat* in the presumptive PGCs (Machado *et al*., 2005).

The DAZ-family of genes encode highly conserved RNA-binding proteins that are essential for gametogenesis in metazoans

The *DAZ* gene family consists of three members that are expressed exclusively in the germ cell lineage. *DAZ genes* are found in a gene cluster on the Y-chromosome of humans and old world monkeys (Yen, 2004). *Dazl* genes are the autosomal paralogs of the *DAZ* genes and are structurally and functionally similar (Xu *et al*., 2001). The *Boule* gene represents a third member of this family and is structurally similar to its relatives retaining the RNA binding motif as well as the unique *DAZ* repeats. While *DAZ* and *Dazl* seem to be required for early germ cell function, *Boule* may be required for sperm formation as homologs have been reported to be restricted tissues of the testis. The *Daz* gene was originally cloned from the AZFc region of Y chromosome from infertile men with azoospermia or severe oligospermia (Reijo *et al*., 1995). *Dazl* is a single copy autosomal gene with essential functions in for the maturation or migration of of PGCs in all organisms described (Maegawa *et al*., 1999; Karashima *et al*., 2000; Reijo *et al*., 2000). In *Drosophila*, Eberhart (1996) illustrated that the *Dazl*-related *Boule* is required for G2/M transition of developing spermatocytes. Experiments performed in *C. elegans* suggest a similar function (Karashima *et al*., 2000). Further study by Houston *et al.,* (1998) showed that *Xenopus Xdazl* can functionally rescue *Drosophila Boule* mutants by overcoming the spermatocyte arrest. These findings suggest that the predetermined mechanism of germ cell formation may be highly conserved even between the protostome/deuterstome lineages.

All *Dazl* family genes encode RNA binding proteins and have the conserved RNA recognition motif (RRM). Recently it has been demonstrated that the Dazla protein in mice

promotes translation of Mvh through a 3'-UTR binding activity and that *Dazla* null mice contain reduced levels of the Mvh protein. Thus, *Dazla*-mediated regulation of *Mvh* mRNA may be a crucial for proper germ cell maturation (Reynolds *et al*., 2005).

Results

Axdazl encodes a protein that is highly related to the product of the mouse Dazla gene

We cloned the *Axdazl* gene to use as a molecular marker for germ cells. This allowed us to compare it with our previously characterized *vasa* homolog, *Axvh*, and see if *Axdazl* expression was consistent with our interpretation that PGC's develop from unspecified embryonic cells. Amino acid sequences present in the products of *Xdazl* (Houston *et al*., 1998), *dazla* (Cook *et al*., 1996) and *Boule* (Eberhart, 1996), were aligned and degenerate oligodeoxynuleotide primers were constructed to regions of high homology in the RNP-1 and RNP-2 domains (RRM). A 220 bp PCR was cloned and used to screen bacteriophage lamda gt11 stage 18 embryo cDNA library (Busse and Sequin, 1993). A total of six positive plaques were purified and the largest of these, 1,545 bp in length, was cloned into pBluescript. The cDNA contained a partial ORF with an in frame stop codon followed by a 958 bp 3' untranslated region and an 18 bp poly-A tail. The 5' end including the start codon was cloned using 5' RACE and the full length coding sequence was assembled and published (GenBank accession no. AF308872). The cloned cDNA is 2037 bp and the deduced protein sequence contains the RRM and the DAZ repeat consistent with all *dazl* ortholgos (Xu et al., 2001). RNA gel blot analysis placed the size of the *Axdazl* transcript at approximately 2.1 kbp, consistent with the sized of our consensus cDNA. The identity of *Axdazl* was verified using sequence comparison with known homologs in the NCBI database as shown in Figure 9. Amino acid sequence of the proteins for *Axdazl* (axolotl), *Xdazl* (*Xenopus*), and *dazla* (mouse) were found by alignment analysis to revealed a surprising degree of similarity between *Axdazl* and *dazla* (70% sequence identity). This similarity was greater than that found between *Axdazl* with *Xdazl* (60% identity), suggesting this gene may not consistent with the organismal phylogeny.

Localization and expression patterns of Axdazl are distinct from that of Xdazl

Xdazl RNA is one of several messenger RNAs that are components of the GP in *Xenopus* oocytes (Houston *et al*., 1998; King *et al.,* 2000). The mechanism of localization in these oocytes is well known (Wilding *et al*., 2001; Chang *et al*., 2004; Choo *et al*., 2005; Wilk *et al*., 2005). GP specific RNAs become associated with the mitochondrial cloud during the early stages of oogenesis. Mediated by sequences in the 3' untranslated regions (3' UTR) (Ikenishi and Tanaka, 2000; MacArthur *et al*., 2000; Chan *et al*., 2001; Machado *et al*., 2005), these RNAs are transported to the vegetal cortex via the METRO RNA localization pathway (Zhou and King, 1996; Kloc *et al*., 2000). We compared the 3' UTR from *Xdazl* (1351 bases) and *Axdazl* (938 bases), and the results, shown in Figure 10, illustrate that the entire 3' UTR of *Axdazl* is conserved within the larger 3' UTR of *Xdazl*. However, the conserved sequences are interspersed with blocks of sequences unique to *Xdazl*. The significance of the *Xdazl* sequence remains to be elucidated.

Because the mitochondrial cloud mediates transports of germ plasm-specific mRNAs to the vegetal cortex, we next asked whether previtellogenic axolotl oocytes contain a mitochondrial cloud. Previtellogenic oocytes dissected from the ovaries of axolotls and *Xenopus* were incubated in the mitochondria-specific florescent dye, and the oocytes were fixed and viewed using fluorescence microscopy or optical sectioning confocal microscopy. Figure 11 shows the resulting images which reveal a localized aggregate of mitochondria (corresponding to the mitochondrial cloud) in previtellogenic stage 1 *Xenopus* oocytes (Fig. 11 panel A and B) An equivalent staining of a mitochondrial cloud-like structure is not present in previtellogenic stage 1 oocytes (Fig. 11, panels C and D). These findings were observed in all of the *Xenopus* oocytes (n=26) axolotl oocytes (n=23) examined. From this data we concluded that axolotl oocytes do not contain a mitochondrial cloud, consistent with previous reports that in contrast to *Xenopus*, the mitochondria of axolotl oocytes are evenly dispersed in the cytoplasm (Wilk *et al*., 2004) and that GP of the type found in the cytoplasm of anuran oocytes is not present in axolotl oocytes.

Localization of Axdazl RNA within axolotl oocytes

Prior to formation of the mitochondrial cloud, *Xdazl* is uniformly distributed in prestage I oocytes(Dumont, 1972). As the *Xenopus* oocyte matures, *Xdazl* co-localizes with

the mitochondrial cloud in a wedge shaped distribution in stage II and III and in the vegetal cortex as early as stage IV. Such a process in axolotl would predict the presence of *Axdazl* mRNA in GP. To test this, we examined *Axdazl* RNA distribution during oogenesis and the results are shown in Figure 12. Sections from adult ovaries hybridized to *Adazl* probe detected strong expression throughout the cytoplasm of pre-stage I oocytes (Beetschen and Gautier, 1989). During vitellogenesis, *Axdazl* RNA-containing cytoplasm froms a complete sphere of central cytoplasm around the nucleus (Fig. 12 D and E), while the yolky peripheral layer shows little stain above background. At early stage IV, *Axdazl* RNA staining has become less intense but is still visible in the central cytoplasm. During later vitellogenesis, yolk granules occupy the entire cytoplasm and little or no staining above background can be observed (fig. 12H). The sensitivity of this assay does not allow us to determine whether maternal *Axdazl* RNA is still present in the mature oocyte. To more precisely exclude localization of *Axdazl* RNA, eight growing oocytes ranging in size from 200μM to 1000μM in diameter were examined in nearly complete serial sections, and in no case was a small region of more intense staining observed. These results demonstrate that GP containing *Axdazl* RNA is not present in axolotl oocytes.

Maternal Axdazl RNA is widely distributed in the axolotl embryo

Sectioned in situ experiments using axolotl oocytes suggest that *Axdazl* is inherited as a maternal transcript (Fig. 12A). Using RT-PCR, we were able to detect low levels of *Axdazl* RNA in the cleavage stage embryos Just prior to the mid-blastula transition (MBT) [stage 9 in axolotls (Masi *et al.,* 2000), as shown in Figure 13. Expression of *Axdazl* decreases after stage 9, but is always present at detectable levels, a pattern that is different from that of *Xdazl* RNA where the RNA disappears after the neurula stage (Housten *et al*., 1998; 2000).

The presence of maternal *Axdazl* mRNA presents the possibility that germ plasm becomes organized in the early embryo and has the potential to direct germ cell determination. We employed RT-PCR to detect *Axdazl* mRNA levels in sectioned axolotl embryos see Figure 14. Much like *Axvh* expression, in blastula stage embryos *Axdazl* is enriched in the animal half, and adjoining region of the vegetal zone, with the highest concentration in the marginal zone (Fig. 14A). At gastrula and neurula stages, *Axdazl* mRNA was detected in all dissected regions. At stages 34 and 40, *Axdazl* RNA is widely

distributed in the trunk and is low or below detection limits in the head (Fig 14. D and E). Finally, at stage 45, when the PGCs have colonized the gonads, *Axdazl* RNA is limited to the posterior middle fraction of the embryo. This same region also contains the developing gonads, a pattern that is similar to that of *Axvh*. These results demonstrate that *Axdazl* RNA is widely distributed throughout the embryo from cleavage to tailbud stages, and is not restricted to the posterior lateral mesoderm, where PGCs arise. By the time PGCs are in the gonads, *Axdazl* mRNA has disappeared from all regions of the embryo except the region that contains the germ cells.

Cell specifc Axdazl expression is restricted to PGCs during migration towards the gonads.

Germ cell expression of *Axdazl* RNA in axolotl oocytes is undetectable in early stage axolotl embryos (pre-tail bud stage embryos). Expression is becomes detectable at stage 33, where labeled cells are located in the dorsal lateral plate in the posterior half of the trunk see Figure 15. In stage 40 embryos, expression of *Axdazl* is localized in germ cells the gonads and next to the mesonepheroi. At this stage *Axdazl*-staining cells also contain nuage. By stage 42, the PGCs have begun to colonize the gonadal region in a more medial position and *Axdazl* labeling becomes evident along with progressively increasing expression levels in later stage embryos, coincident nuage accumulation kinetics. Taken together our analyses of *Axdazl* expression patterns support our conclusion that the onset of embryonic *Axdazl* gene expression in germ cells coincides closely with the migrating PGCs and with the appearance of nuage. The diffuse appearance of *Axdazl* mRNA stage 33 embryos appears to be of maternal origin.

Discussion

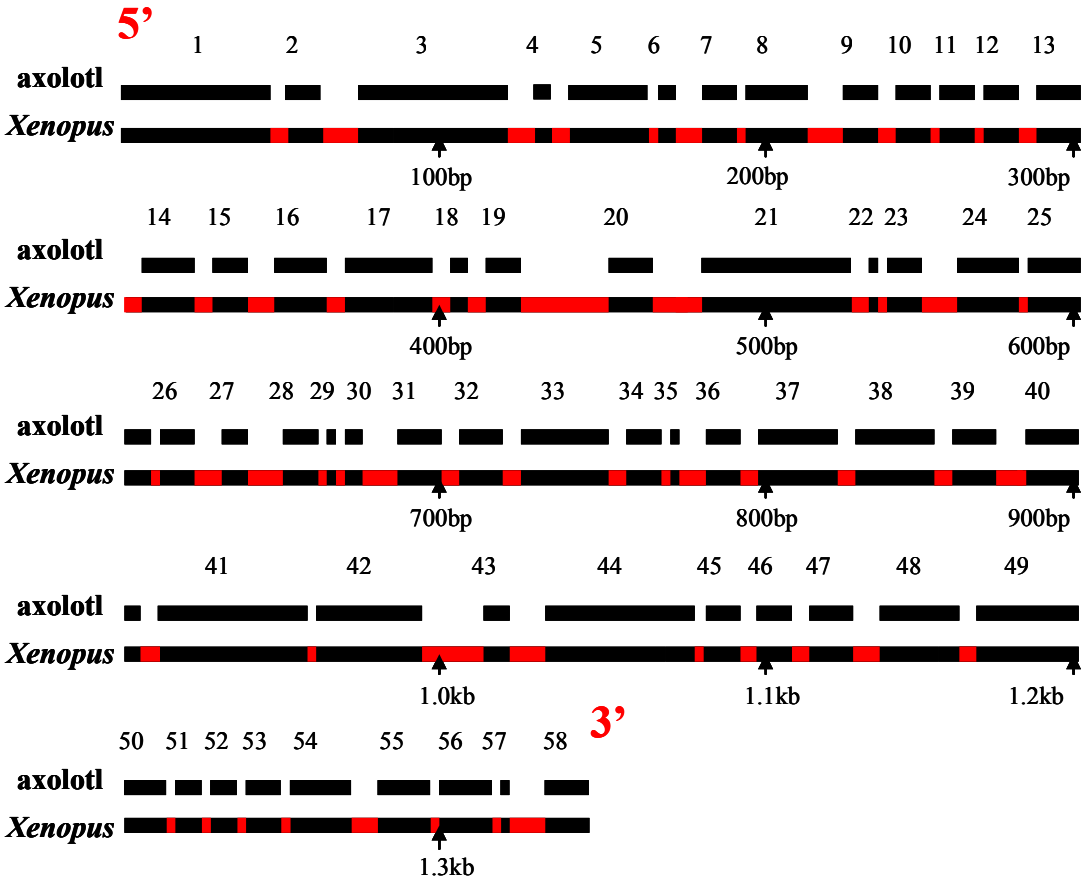
Xdazl RNA is associated with other components of GP that are required for germ cell formation in *Xenopus*, and we have used it as a marker to search for GP in axolotls. We have found that *Axdazl* RNAs distributed throughout the cytoplasm of previtellogenic and early vitellogenic oocytes, rather than localized as is *Xdazl* RNA in *Xenopus*. In larger oocytes and early embryos, the sensitivity of the in situ hybridization method was not sufficient to detect *Axdazl* RNA, but the absence of any localized staining indicates that it is diffuse, presumable due to dilution during oocyte growth. [*Xdazl* RNA is clearly detected by similar methods in

the GP of large *Xenopus* oocytes (Houston *et al*., 1998)]. These results are consistent with the absence of a mitochondrial cloud, which mediates formation of the GP in the vegetal region of frog oocytes. Taken together, we provide strong evidence that GP of the type found in anuran eggs is not present in the vegetal pole of axolotl eggs.

If GP were to exist in axolotl eggs and early embryos, it would most likely be localized to the marginal zone cytoplasm from which the PGCs are derived. Interestingly, in the axolotl blastula, *Axdazl* RNA is most concentrated in the marginal zone but also extends throughout the animal hemisphere, similar to the expression of *Axvh* (Fig. 6). By stage 10.75 expression of *Axdazl* RNA is found quite equally distributed in all regions of the embryo with similar concentrations in the ventral and dorsal marginal zones. Based on these results, it is unlikely that GP components are sufficient for germ cell formation in the axolotl, since they appear to be in low abundance and are widely distributed in the early embryo, rather than localized to the germ cell-forming region.

```
                                                   RNP-2
Xdazl       MSGKEESSNYAATAEEEAVN-------QGFVLPEGEIMPNTVFVGGIDITMDEIEIRDFF 53
                                          |||||||||||| |||||||||
Axdazl                                    MPNTVFVGGIDIRMDEIEIRDFF 23
                                          |||||||||||: ||| ||| ||
dazla       MSATTSEAPNSAVSREASTQSSSATTSQGYVLPEGKIMPNTVFVGGIDVRMDETEIRSFF 60

                            RNP-1
Xdazl       TRFGNVKEVKIITDRTGVSKGYGFISFSDEVDVQKIVKSQISFHGKKLKLGPAIRKI--- 110
             ||| |||||||||||||||||:|| |:||||||| ||| |||||||||||||
Axdazl      ARFGAVKEVKIITDRTGVSKGYGFVSFHDDVDVQKIVDSQINFHGKKLKLGPAIRKQQNL 83
            ||:|:|||||||||||||||||||||| :|||||||||:||||||||||||||||||| ||
Dazla       ARYGSVKEVKIITDRTGVSKGYGFVSFYNDVDVQKIVESQINFHGKKLKLGPAIRKQ-NL 119

                                                          DAZ Repeat
Xdazl       -CTYVQPRPVVLS--HPTPFHHAWNNQNADS-YIQHSPIVSPITQYVQACPYPSSPPMAI 166
               :||||| ||       ||  |  |||:: |:||  ::||: ||| | || ||||  |
Axdazl      CAYHVQPRPMVLN-PPAPQFHSMWSSQNAETYYMQHPHMISPVPQYVPAYPYQSSPPVII 142
            | |||||||:::| || |||:| ||| |||| ||||| |: |: ||| |||   |
dazla       CTYHVQPRPLIFNPPPPPQFQSVWSSPNAET-YMQPPTMMNPITQYVQAYPPYPSS---P 175

Xdazl       QQIPVGCQQPGY-FQVSPQWPAD-QRSYMFPTPAFTFNYHCCDMDPNGGEPIPREYPIDQ 224
            |||||| ||| | :|:||||    || |::|    : |||| ::|  | | |  |  : :
Axdazl      QQIPVGYQQPTYNYQLSPQWAPGEQRGYIIPQAYAAVNYHCTELDQPGAELIQAEFPVHE 202
            |:  ||| | ||||: |||  |||| |:|| ||::|||||:|:| |||: :  |  | :
dazla       VQVITGYQLPVYNYQMPPQWPAGEQRSYVIPPAYTTVNYHCSEVD-PGADILPNECSVHD 234

Xdazl       -TVSASGANPQKRYVEMSTQTIVSCLFDPANKFH--SFVSQEDYLKDNRVHHLRRRESVI 281
            :  :||  |||: |: | ||:||||| | |::   ||||||:|:||:|||::||  :|:
Axdazl      PAQPSSGNSPQKKSVDRSIQTVVSCLFTPENRLPRNSFVSQEEYMKDKRVHQFRRSKAVF 262
            |  :||| |||||||||||||||||| |||||  ||:|:|::|:||||||:|||| ||:
Dazla       -AAPASGNGPQKKSVDRSIQTVVSCLFNPENRLR-NSLVTQDDYFKDKRVHHFRRSRAVL 292

Xdazl       KRVSK- 286
            |
Axdazl      KTVPN- 267
            |
dazla       KSDHLC 298
```

Figure 9. Amino acid sequence comparison of the products of *Axdazl* (axolotl) with *Xdazl* (*Xenopus*) and *dazla* (mouse). The deduced amino acid sequences of the axolotl (*Axdazl*), *Xenopus* (*Xdazl*) and mouse (*dazla*) *DAZ*-like gene products are aligned. *Axdazl* encodes a protein that is 70% identical and 81% similar to the product of *dazla*, and 60% identical, 71% similar, to the product of *Xdazl*. *Xdazl* is 59% identical, 77% similar to *dazla*. RNP-1 and RNP-2 binding domains conserved in other *DAZ*-like proteins, and a region homologous to the *DAZ* repeat found in the human Y-linked *DAZ* gene are indicated by bold lettering. Identical amino acids are indicated by vertical lines. Conservative substitutions are indicated by dotted lines.



1-	53%	42bp	13-	64%	11bp	25-	85%	20bp	37-	52%	27bp	49-	53%	47bp
2-	80%	10bp	14-	67%	15bp	26-	82%	11bp	38-	58%	31bp	50-	70%	13bp
3-	61%	51bp	15-	78%	9bp	27-	100%	6bp	39-	54%	13bp	51-	83%	6bp
4-	100%	7bp	16-	82%	16bp	28-	90%	10bp	40-	56%	18bp	52-	88%	7bp
5-	63%	24bp	17-	63%	32bp	29-	75%	4bp	41-	53%	45bp	53-	78%	9bp
6-	67%	6bp	18-	60%	5bp	30-	75%	4bp	42-	68%	25bp	54-	64%	21bp
7-	64%	11bp	19-	82%	11bp	31-	89%	9bp	43-	78%	9bp	55-	58%	19bp
8-	70%	20bp	20-	85%	8bp	32-	58%	12bp	44-	52%	45bp	56-	63%	16bp
9-	67%	9bp	21-	62%	49bp	33-	58%	33bp	45-	56%	9bp	57-	100%	3bp
10-	86%	7bp	22-	100%	4bp	34-	90%	10bp	46-	67%	12bp	58-	100%	12bp
11-	86%	7bp	23-	67%	12bp	35-	100%	3bp	47-	53%	17bp			
12-	57%	7bp	24-	55%	20bp	36-	56%	9bp	48-	57%	35bp			

Figure 10. Sequence organization within the 3' UTR of *Axdazl* and *Xdazl*. (A). The 3' untranslated regions (3' UTR) within the mRNAs encoding *Axdazl* and *Xdazl* are aligned. The entire *Axdazl* 3'UTR is represented on the top line. Spaces indicate positions that do not align with the *Xenopus* sequence. On the bottom line is the *Xdazl* 3' UTR. Black segments indicate regions with homology to the axolotl sequence. Gray segments indicate unique regions of sequence that do not align with the axolotl 3' UTR. (B). Shows the length of each segment of conserved sequence and the percentage of identical bases.

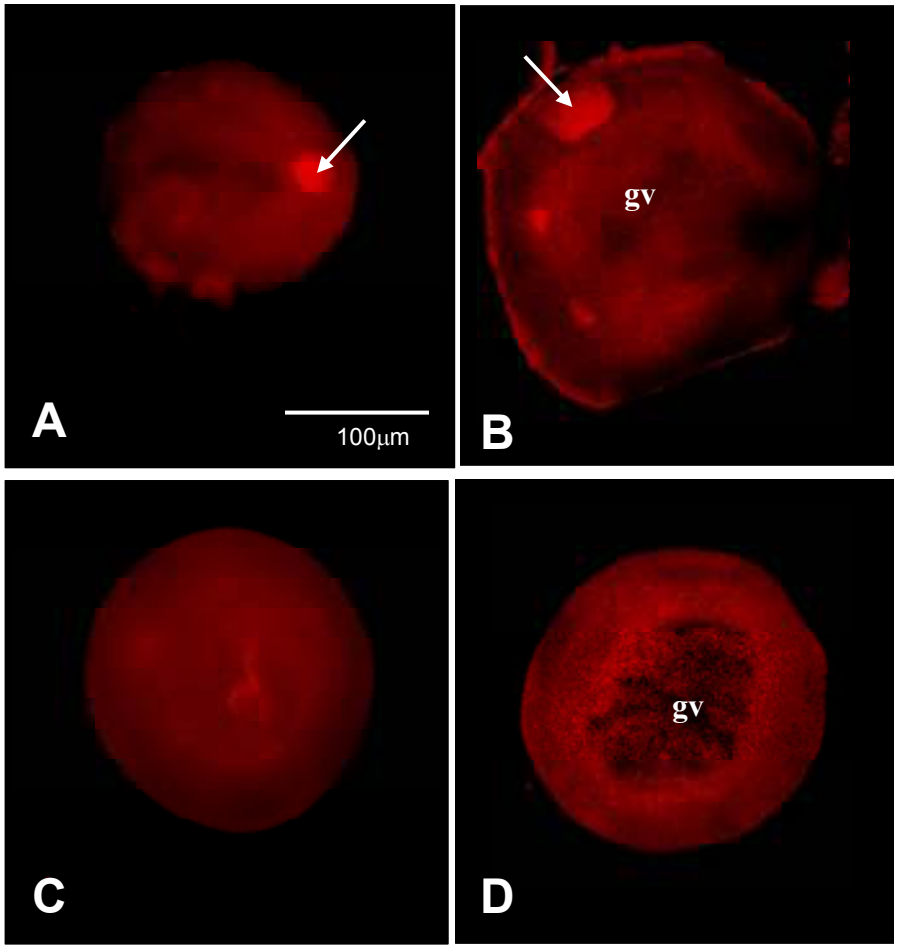


Figure 11. Previtellogenic axolotl oocytes do not have a mitochondrial cloud. Previtellogenic axolotl oocytes ranging from 170 to 390 mm in diameter (Stage 1, Beetschen and Gautier, 1989), and *Xenopus* oocytes ranging from 140 to 290 mm (Stage 1 to 3 Dumont, 1972) were incubated in MitoTracker Red and examined either by Quantitative Fluorescence Microscopy (A,C) or as optical sections with a confocal microscope (B,D). (A,B). Examples of *Xenopus* oocytes aggregates of mitochondria corresponding to the mitochondrial cloud (arrow). (C,D). Examples of axolotl oocytes lacking a mitochondrial cloud.

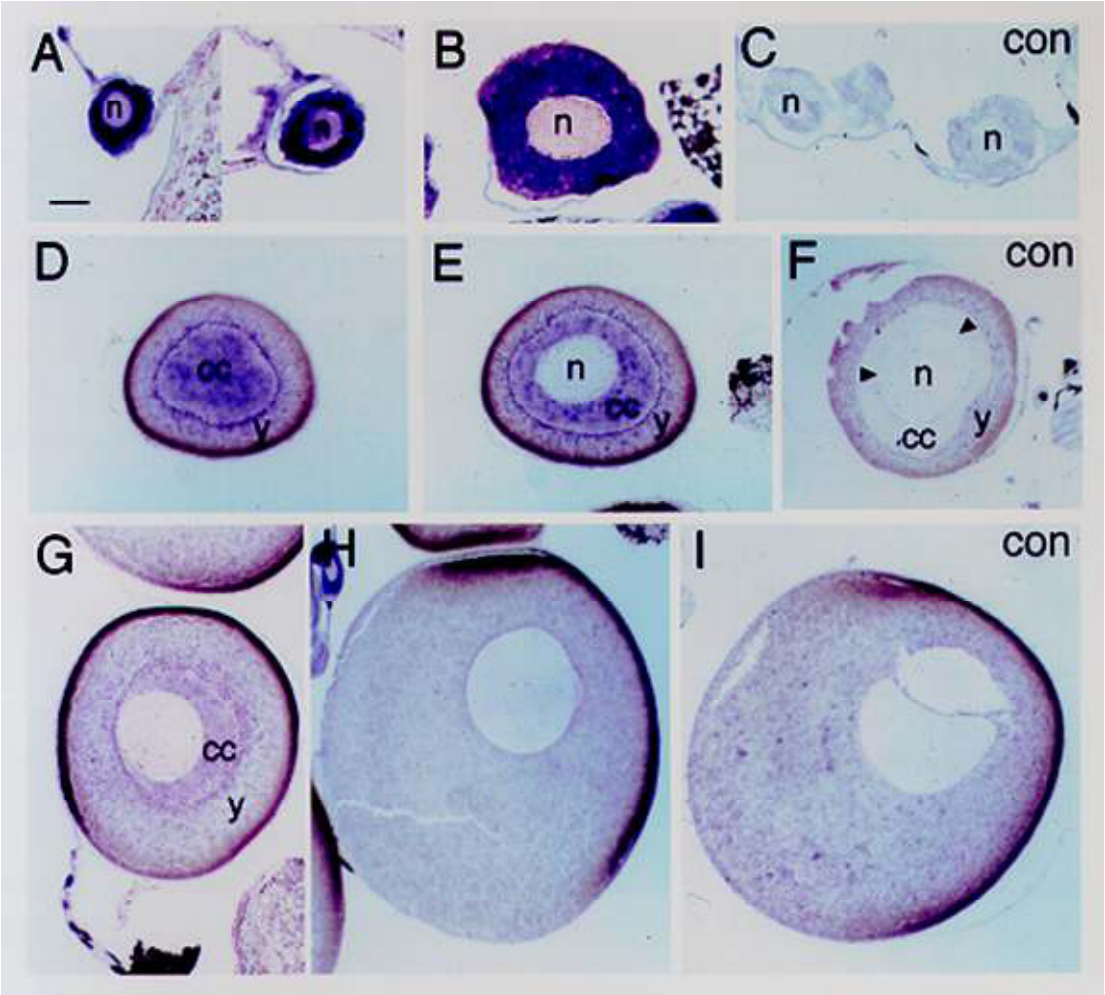


Figure 12. *Axdazl* RNA is not localized in the cytoplasm of oocytes. Sections of fragments of adult ovaries were hybridized to an antisense *Axdazl* digoxigenin-labeled probe except where indicated. (A). Two different small stage I oocytes showing a ring of dark staining cytoplasm surrounding the large pale nucleus. (B). A larger stage I oocyte showing stained cytoplasm surrounding the pale nucleus. This section was counterstained with safranin. (C). Section hybridized to a control sense *Axwnt-8* probe, showing two small stage I oocytes (arrows) with little cytoplasmic stain. (D,E). Sections of the same stage III oocyte. (D). The section passes through the central blue stained cytoplasm, but not the nucleus. (E). The section shows the central blue stained cytoplasm as a ring around the pale staining nucleus. Comparison with F shows that the *Axdazl* staining is primarily in the central layer of cytoplasm surrounding the nucleus. (F). A stage III oocyte hybridized to the sense probe. The nucleus (arrowheads) is surrounded by a strip of pale central cytoplasm and an outer strip of darker yolky cytoplasm. (G). A stage IV oocyte. The yolky layer takes up most of the cytoplasm. Light staining is still seen in the central cytoplasm surrounding the nucleus. (H). A stage V oocyte 1650 mm in diameter, showing little staining above background. Fully grown oocytes at stage VI are about 2000 mm in diameter. (I). A stage V oocyte hybridized to the sense probe. Scale bar: 100 mm (A-C); 200 mm (D-I). cc, central cytoplasm; n, nucleus; y, yolky layer of cytoplasm.

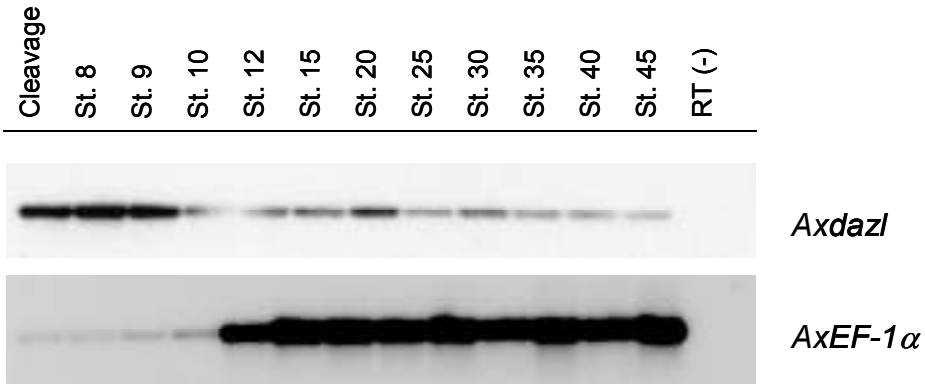
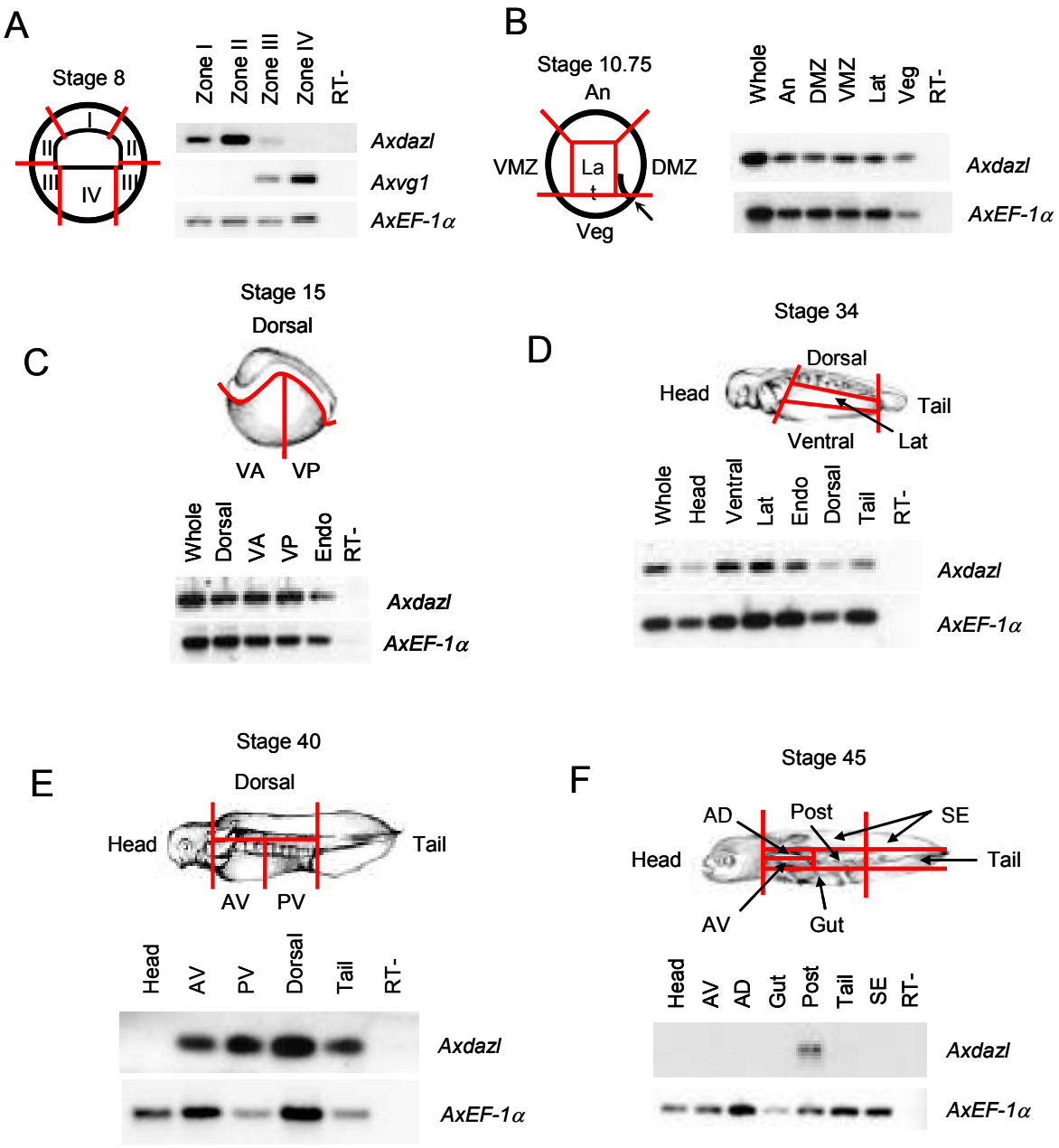


Figure 13. *Axdazl* RNA is expressed throughout embryogenesis. RNA was extracted from groups of 5 embryos at each of the indicated stages. An equivalent amount of RNA from each stage was reverse transcribed. Portions of each reaction were used in PCR to amplify the indicated sequences within the linear range. Products were detected by hybridization of labeled cloned sequences to Southern blots. Maternal *Axdazl* RNA is inherited by embryos, and levels begin to decline by stage 10. Low levels of RNA are found in embryos through development to stage 45. *EF-1α*, which becomes transcriptionally active at the mid-blastula transition is included as a control.

Figure 14. *Axdazl* RNA shows widespread distribution in embryos. Ten embryos from each of the indicated stages were dissected into small parts as indicated on the figure. RNA was extracted from each region and reverse transcribed. Input cDNA was normalized by RNA input and EF-1α levels. The indicated sequences were amplified by PCR and detected as described in Methods (Chapter 5). (A). Stage 8 embryos. Regions I,II,III, and IV corresponding to the animal pole region, the peripheral region of the animal cap including the animal portion of the marginal zone, vegetal region of the marginal zone, or vegetal pole, respectively, were prepared as described by Nieuwkoop (1969). (B). Stage 10.75 embryos. Fractions are as follows: Whole (whole embryo); An (animal pole); DMZ (dorsal marginal zone); VMZ (ventral marginal zone); Lat (lateral marginal zone); Veg (vegetal pole). (C). Stage 15 embryos. Fractions are as follows: Whole (whole embryo); Dorsal; VA (ventral anterior); VP (ventral posterior); Endo (endoderm). (D). Stage 34 embryos. Fractions are as follows: Whole (whole embryo); Head; Ventral; Lat (Lateral); Endo (Gut Endoderm); Dorsal; Tail. (E). Stage 40 embryos. Fractions are as follows: Head; AV (anterior ventral); PV (posterior ventral); Dorsal; Tail. (F). Stage 45 embryos. Fractions are as follows: Head; AV (anterior ventral mesoderm); AD (anterior dorsal mesoderm); Gut (gut tube); Post (posterior ventral mesoderm); Tail; SE (surface ectoderm). An RT (-) lane showing the product of a reaction performed with RNA from a whole embryo that was not reverse transcribed is included for each stage as a negative control . *Axdazl* RNA expression is widespread in early embryos, but is specific to the posterior ventral mesoderm, which includes the gonads, by stage 45.


A
Stage 8
I
II
II
III
III
IV
Zone I
Zone II
Zone III
Zone IV
RT-
Axdazl
Axvg1
AxEF-1α
B
Stage 10.75
An
VMZ
La
t
DMZ
Veg
Whole
An
DMZ
VMZ
Lat
Veg
RT-
Axdazl
AxEF-1α
C
Stage 15
Dorsal
VA
VP
Whole
Dorsal
VA
VP
Endo
RT-
Axdazl
AxEF-1α
D
Stage 34
Dorsal
Head
Tail
Ventral
Lat
Whole
Head
Ventral
Lat
Endo
Dorsal
Tail
RT-
Axdazl
AxEF-1α
E
Stage 40
Dorsal
Head
Tail
AV
PV
Head
AV
PV
Dorsal
Tail
RT-
Axdazl
AxEF-1α
F
Stage 45
AD
Post
SE
Head
Tail
AV
Gut
Head
AV
AD
Gut
Post
Tail
SE
RT-
Axdazl
AxEF-1α

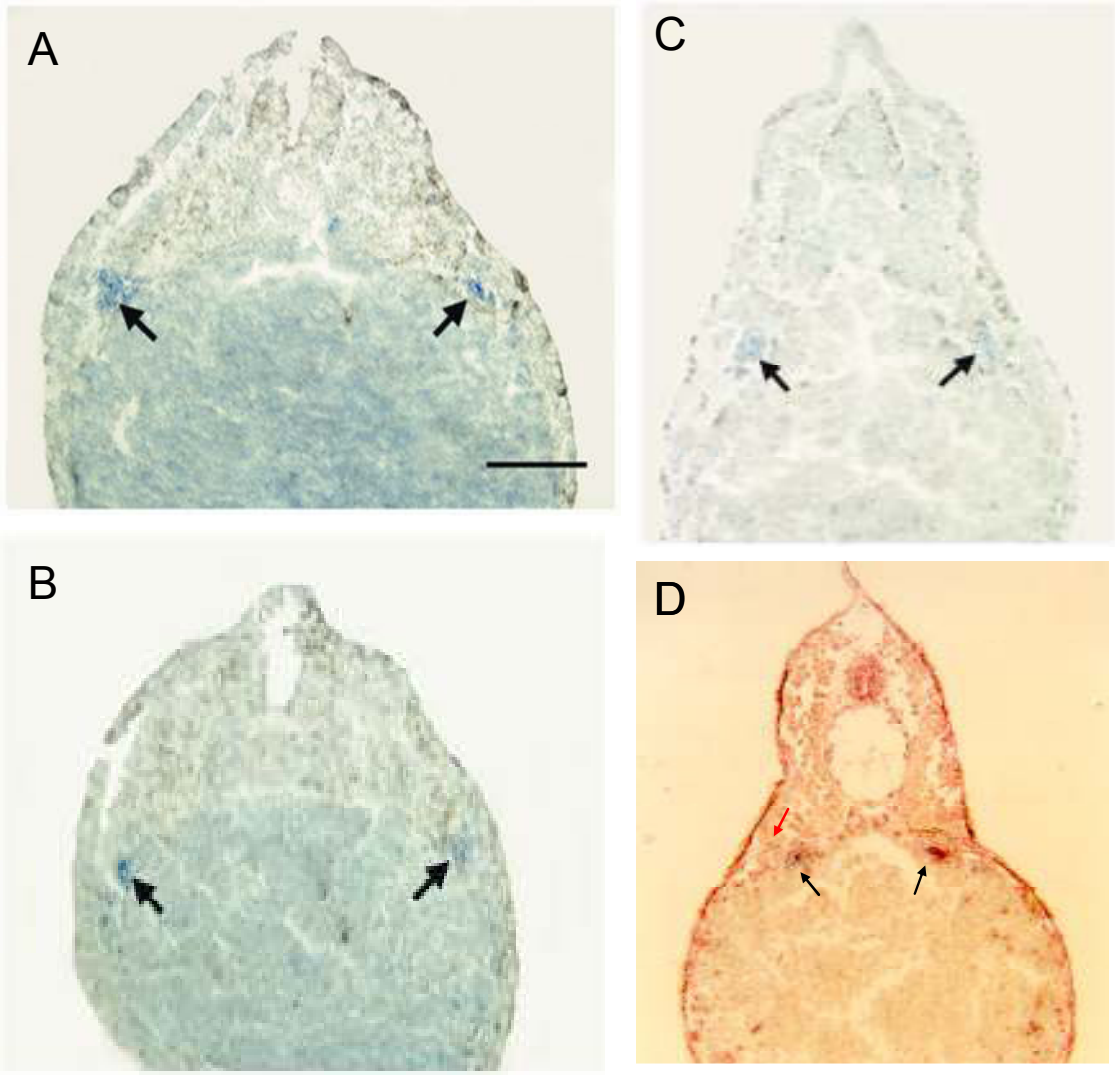


Figure 15. Expression of *Axdal* in axolotl embryos, cross sections of the posterior region. A. The earliest detection of *Axdazl* expression in germ cells is stage 33. Arrows mark the germ cells migrating through the dorsal edge of the lateral plate. B. A more posterior section of the same embryo (stage 33), showing *Axdazl* specific expression. C. Stage 36 embryo hybridized to *Axdazl*. D. Posterior section of a stage 42 embryo showing Axdazl expression approaching the developing gonad. Scale bar in A= 200μM.

CHAPTER 4

EVOLUTON OF THE REGULATIVE GERM CELL FORMING MECHANISM IN TERRESTRIAL VETEBRATES

Background

Over 100 years ago, Weismann (1885) proposed the concept that germ plasm, (GP) a specialized region of egg cytoplasm, would contain sufficient material to direct the establishment of the germ cell lineage during embryonic development. Since that time, GP has been identified in the eggs of many species from both the protostomal and deuterostomal lineages (Kloc *et al.,* 2001; Mahowald 2001). As suggested by Weismann, GP contains molecules that act as determinants to specify germ cells. The progeny of cells inheriting the GP eventually give rise to PGCs, the founder cells of the germline, which later move to the gonad and develop into gametes (Houston & King 2000*a*; Kloc *et al.,* 2001). The eggs of many of the non-mammalian model systems used to study animal development contain GP. For this reason it has long been assumed that GP and the process of cell-autonomous germ cell specification that it governs, is a mechanism that has been evolutionarily conserved as part of the developmental program in all animal embryos. However, experiments in recent years with mouse embryos suggest otherwise.

In mouse embryos GP has never been identified, and the presumptive germ cells are clonally related to somatic cells, indicating that mouse PGCs are not specified cell-autonomously (Eddy 1974, 1975; Lawson *et al.,* 1999; Ying & Zhao 2001; Ying *et al.,* 2001). From these studies it is clear that mouse PGCs are not specified by maternal germ cell determinants, making them different from other animal models. In addition, extracellular signaling is clearly responsible for allocating cells to the germline during mouse development. Several studies have demonstrated that members of the TGF-b family of signaling molecules secreted from adjacent extraembryonic tissues are required for the production of PGCs in mouse embryos.

One of the major concepts fostered by modern developmental biology is that most events occurring during animal development are evolutionarily conserved across vast phylogenetic distances. For example, *Hox* genes regulate in axial patterning in a diverse array of animals (reviewed Hoegg and Meyer, 2005). However, the mechanism of germ cell specification in mouse embryos is different from that in frog embryos. The lack of a GP mode of germ cell specification in mouse was consistent with the interpretation that the mouse and mammalian inductive mode was evolutionarily derived. However, it is also possible that the GP mode is more derived while the inductive mode of the mouse is more ancestrally conserved.

To understand the evolution of developmental mechanisms in mammals, it is important to recognize that all terrestrial vertebrates share a common neotetrapod ancestor (Cannatella & Hillis 1992; Milner 1992). Therefore, all developmental processes in tetrapod embryos have either diverged or been conserved from a single ancestral mechanism. From the ancestral tetrapod, two major lineages of modern amphibians have evolved: anurans (frogs) and urodeles (salamanders). The embryos of each of these lineages have been extensively used in experimental studies dating back over a century, and basic aspects of the process of PGC development have been elucidated (reviewed Hanken, 1984).

Anurans and urodeles develop PGCs in very different ways. In urodele embryos, the PGCs develop in the lateral plate mesoderm from cells fate mapped to the ventral marginal zone of gastrula stage embryos (Humphrey 1925, 1929; Ikenishi & Nieuwkoop 1978) (Nieuwkoop 1947). In the absence of GP, it is unclear how presumptive germ cells are distinguished from somatic cells developing in adjacent domains, such as the primitive blood. However, in his classical mesoderm induction studies, Nieuwkoop (1969) showed that mesoderm inducing signals produced from vegetal blastula stage cells (presumptive endoderm) could induce ectopic PGCs from presumptive ectoderm, (this dissertation, Chap. 1, Fig. 3). Later work further distinguished the signals needed to produce PGCs, showing that they are derived from ventral vegetal cells (Boterenbrood & Nieuwkoop 1973; Sutasurya & Nieuwkoop 1974). Yet, these same signals also produce a wide variety of somatic ventral and lateral cell types, including blood, mesonephros and mesothelium. From these studies it can be concluded that, unlike anurans, in urodele embryos PGCs do not develop from predetermined cells. Rather, urodele PGCs appear to develop from unspecialized

mesodermal tissue that has responded to extracellular inducing signals from the vegetal hemisphere. And these specific agents are sufficient to convert the presumptive ectodermal tissue to a complex array of mesodermal cell types. Among the cell types induced within the animal moiety of the embryo were PGCs. Since the time of Nieuwkoop's experiments, work with *Xenopus* embryos has established that the inducing agents secreted from the cells of the vegetal hemisphere in amphibians are growth factors representing just a few major families. These can be introduced into embryos as exogenous agents, by microinjection of synthetic RNAs from cloned templates, and they are capable of inducing mesoderm in isolated animal cap explants (Jones & Smith 1999). The character of the mesoderm produced in these experiments can be controlled either by introducing specific agents or by altering the levels of these agents. Moreover, factors can be used alone or in combination to produce mesoderm of dorsal, ventral or intermediate type. Among the endogenous growth factors identified in *Xenopus* embryos is eFGF. FGF signalling is required to produce posterior mesoderm in normal embryos (Amaya *et al.,* 1991). eFGF can induce mesoderm in animal cap explants. BMPs represent another growth factor family expressed in *Xenopus* (Dale & Jones 1999). BMPs are expressed across the dorsal–ventral (D/V) axis of *Xenopus* embryos. However, BMP antagonists secreted from the dorsal organizer (Spemann organizer) establish a gradient of BMP activity, with high levels of BMP activity on the ventral side and low levels on the dorsal side (Jones & Smith 1998). These, in turn, pattern the mesodermal layer. High levels of BMP activity induce the production of ventral mesoderm, and dorsal mesoderm is produced in response to the low BMP activity on the dorsal side.

Fundamentally, for any newly acquired trait to evolve it requires that the trait itself is genetically transmissible. In this regard, the sequences of the genes that govern PGC determination may provide insights into how germ cell-determining mechanisms evolved. The usefulness of DNA sequence analysis as a method for gauging species relatedness is widely recognized. In general, the relatedness of sequence between genes of any given species is a reflection of the order of species relatedness. Thus, because urodeles and anurans are sister taxa (i.e. they are both lissamphibians), any gene would be expected to show greater similarities between these groups than with less related groups, such as mammals. Johnson *et al.,* (2003) tested this hypothesis with several genes known to regulate early embryogenesis, *GATA-2*, *Brachyury* and *goosecoid*, and in each case the genes reflect

the phylogenetic afinites, with axolotl and *Xenopus* being highly related. However, similar analyses on genes that are known to regulate germ cell determination and showed quite different results. Preliminary phylogenetic analysis, using limited sequence data, in Johnson *et al.,* (2003) suggest that the genes that code for proteins required in germ cell formation show a mechanistic gene phylogeny, with orthologs of *Oct-4* and *dazl* showing a distinct relationships between organisms with similar germ cell forming mechanisms even across wide evolutionary distances. Through phylogenetic analysis of genes that govern germ cell development, Johnson *et al.,* (2003) demonstrated that mammals, urodeles and lungfish can be grouped into a class of animals (sarcopterygians), whose embryos retain an ancestral mode of germ cell development in addition to retaining primitive embryological features.

We have proposed that regulative germ cell specification is ancestral to tetrapods, and that germ plasm has evolved independently several different times, in several different lineages (Johnson *et al*., 2001, 2003). Here we present results indicating that axolotl PGCs are produced from naïve mesoderm in response to simple mesoderm-inducing agents, and extend these findings by cladistic analysis of *vasa* homologs from a variety of vertebrates. In this analysis we also include a *vasa* homolog from member of a basal sacropterygian to serve as an outgroup for phylogenetic character mapping, the lungfish *Prototerus annectans*. Phylogenetic analysis suggested that germ cell determination in mice and axolotls are likely due to a conserved ancestral mechanism rather than convergence on a similar process (Johnson *et al*., 2003). The findings are discussed with respect to how and why different modes of germ cell determination have evolved in the vertebrates.

Results

PGC induction by eFGF and BMP-4

Expression of BMP-4 acts as a developmental regulator, BMP-4 causes ventral mesoderm to be produced (Dale *et al.* 1992; Jones *et al.,* 1992). Animal caps isolated from *Xenopus* embryos that are injected with RNA encoding eFGF and BMP-4 express high levels of the embryonic globin gene, β-globin, a marker of primitive blood (Mead *et al.,* 1998). Because in axolotls primitive blood develops adjacent to the germ cell precursors (Nieuwkoop 1947), we asked whether the combination of eFGF and BMP-4 is sufficient to direct the induction of PGCs in isolated axolotl animal caps. Axolotl embryos were injected

at the one-cell stage with 5ng each of RNA encoding *Xenopus* eFGF and BMP-4 as shown in Figure 16. Control embryos were injected with an equal volume of water. The injected embryos were cultured in amphibian saline media until the early blastula stage after which, the animal caps were harvested at an early larval stage (stage 43). This stage was chosen because of previous reports that PGCs become identifiable in animal caps cultured until about this stage (Ikenishi and Nieuwkoop, 1978). Also, the PGC specific molecular marker *Axdazl* is strongly expressed by this stage (this dissertation, Chap. 3, Fig. 14). Harvested animal caps were probed for germ cell-specific *Axdazl* expression by whole-mount *in situ* hybridization. In two experiments we detected *Axdazl* expression (arrows) in all of animal caps isolated from embryos injected with eFGF and BMP-4 RNAs (Experiment 1: 7/7; Experiment 2: 5/5; Fig. 16, panel B). *Axdazl* expression was not detected in caps from control embryos (Experiment 1: 0/5; Experiment 2: 0/5). These results indicate that PGCs can be induced from axolotl animal caps by simple combinations of defined growth factors.

The evolution of regulative germ cell specification

The production of germ cells is among the most critical events in early development, since in the absence of germ cells an organism's lineage will cease to exist. Thus, the existence of highly variable mechanisms of germ cell development appears to present a paradox, since most events of early development have been highly conserved throughout the animal kingdom. To consider a unifying paradigm to explain the divergence of germ cell determining mechanisms, we examined the distribution of species with and without predetermined germ cells in deuterostomes. We concluded that species with predetermined germ cells are associated with derived cell movements during gastrulation, leading to derived adult morphologies (e.g. adult frog morphology)(Johnson et al., 2003). Conversely, species in which germ cells form in response to inducing signals develop from highly conserved gastrulation movements and have more primitive adult morphologies [e.g. adult salamander morphology (A summary of the conclusions is presented in figure 17)]. They concluded that the most likely hypothesis is that GP evolved independently in several different lineages of deuterostomes. In addition they propose that regulative germ cell specification is primitive, whereas the predetermined mode is derived. As such, among terrestrial vertebrates, anurans as well as avians (Tsunekawa *et al.,* 2000) independently evolved GP and determinative

germ cell specification. Mammals and urodeles, on the other hand, have retained the primitive mechanism.

Phylogenetic Analysis of the PGC-associated Gene vasa

Several genes that regulate germ cell development in diverse species have now been identified. The gene encoding *Axvh* is expressed in axolotl PGCs (this dissertation, Chap. 2), and homologs of this gene are also all germ cell specific. In addition, full length sequences have also been reported in a number of vertebrates, making *vasa* an attractive gene for phylogenetic analysis. To extend this analysis, we isolated the homologue of *vasa* from the African lungfish, *Protopterus annectans*, and named the gene *Provh (Protopterus vasa homolog)*. *Protopterus* was chosen because it has primitive adult and embryonic features, and because phylogenetic information indicates that it is likely to be among the extant species that is most closely related to the ancestral tetrapod (Zardoya *et al.,* 1998). *Provh* was cloned using degenerate oligonucleotide PCR, and the PCR product was used to isolate a full-length clone from a cDNA library. The cDNA and protein sequence are shown in Figure 18 along with a conserved domain diagram showing the location of the diagnostic DEAD and helicase domains. Confirmation of germ cell specificity of *Provh* was investigated using northern blot analysis, see figure 19).

Protein product of *Provh*, *Axvh* and other *Vasa* orthologs were arranged using clustalw (http://www2.ebi.ac.uk/clustalw/) and further aligned by inspection. The amino acid data set was used for the construction of a maximum parsimony (MP) tree using PAUP 4.0 (Swofford, 1998). Figure 20 shows the results of the *vasa* analysis using proteins encoded from germ cell-specific *vasa* orthologs from a variety of different vertebrates as listed in Table 1. The results demonstrate that the sequence of a protein encoded by a germ cell specific gene does not track with species phylogeny, as other genes do (see above). Thus, rather than recover the standard amphibian species relationship, the analysis of Vasa related proteins recover a close lungfish + salamander + mammal relationship. When these data are considered with respect to the correlation of regulative germ cell development in animals with primitive embryological development as seen in figure 17, they strongly support the likelihood that determinative germ cell development in Anura is a independently derived.

Table 1. Vasa orthologs from various species

Gene	Organism	Accession No	Reference
Vasa	*Drosphila*	M32560	Hay *et al*., 1988
DDX4	*Homo*	NM010029	Castrillon *et al*., 2000
Axvh	*Ambystoma*	AY542375	Bachvarova et al., 2004
Mvh	*Mus*	D14859	Fujiwara *et al.,* 1994
XVLG1	*Xenopus*	AF190623	Komiya *et al.,* 1994
RVLG	*Rattus*	s75275	Komoya *et al.,* 1995
RIVLG	*Rana*	AJ841700	Marracci *et al.*
Provh	*Protopterus*	Not in database	In this study
Stvh	*Acipenser*	Not in database	In prep
Zvh	*Danio*	AB005147	Yoon *et al*., 1994

Discussion

We proposed that regulative germ cell specification is primitive to the vertebrate lineage (Johnson et al., 2001; 2003). This implies directly that determinative germ cell specification, as observed in zebrafish, anuran amphibians and chicks, evolved independently in each of the respective lineages. This hypothesis refutes the long-standing belief that determinative germ cell specification is an ancestral conserved process, shared within the deuterostomal and protostomal lineages. We consider the appearance of GP in the embryos of divergent lineages of animals to be an example of convergent evolution. To suggest convergent evolution of a process or a structure demands that it must be acted upon favorably by the pressures of natural selection. The end result of natural selection is to select for evolutionary events that enhance an organism's ability to reproduce, i.e. to pass genetic material to a new generation through the germline. It is also reasonable to assume that the specification of germ cells by preexisting maternal molecules, i.e. GP, occurs much earlier in development than when PGCs are specified by extracellular signals, and this would probably allow accelerated embryogenesis. In addition, germ cells specified by maternal molecules are likely to be less sensitive to changes in developmental signaling, which could create conditions permissive for embryos to evolve novel signaling mechanisms that further accelerate development. In this regard, it should be noted that the embryos of anuran amphibians and teleost fishes, both of which contain GP, develop considerably faster than their sister taxa with regulative germ cells.

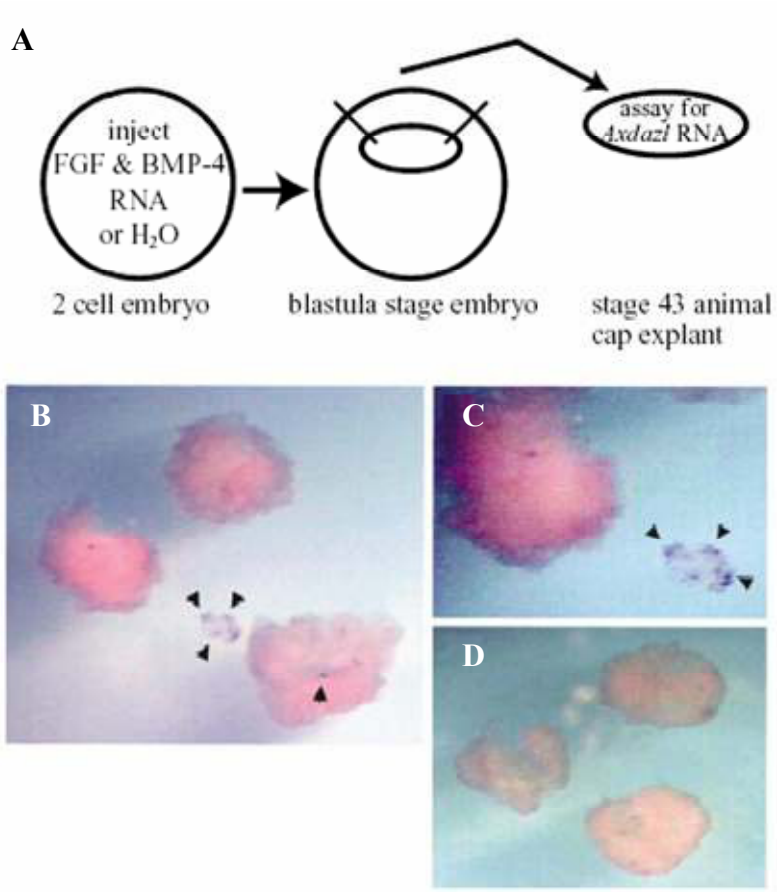


Figure 16. Induction of PGCs in isolated axolotl animal caps A. Schematic diagram showing experimental protocol. B. Animal caps injected with eFGF and BMP-4 RNAs, probed for *Axdazl* expression by whole-mount *in situ* hybridization, using digoxigenin labelled *Axdazl* antisense RNA as a probe (see Materials and Methods, chapter 5). Arrows indicate *Axdazl* positive cells. *Axdazl* expressing cells are always produced in clusters in these experiments. C. Same figure as in (*B*), magnified. D Animal cap from embryo injected with H_2O. *Axdazl* expressing cells are absent.

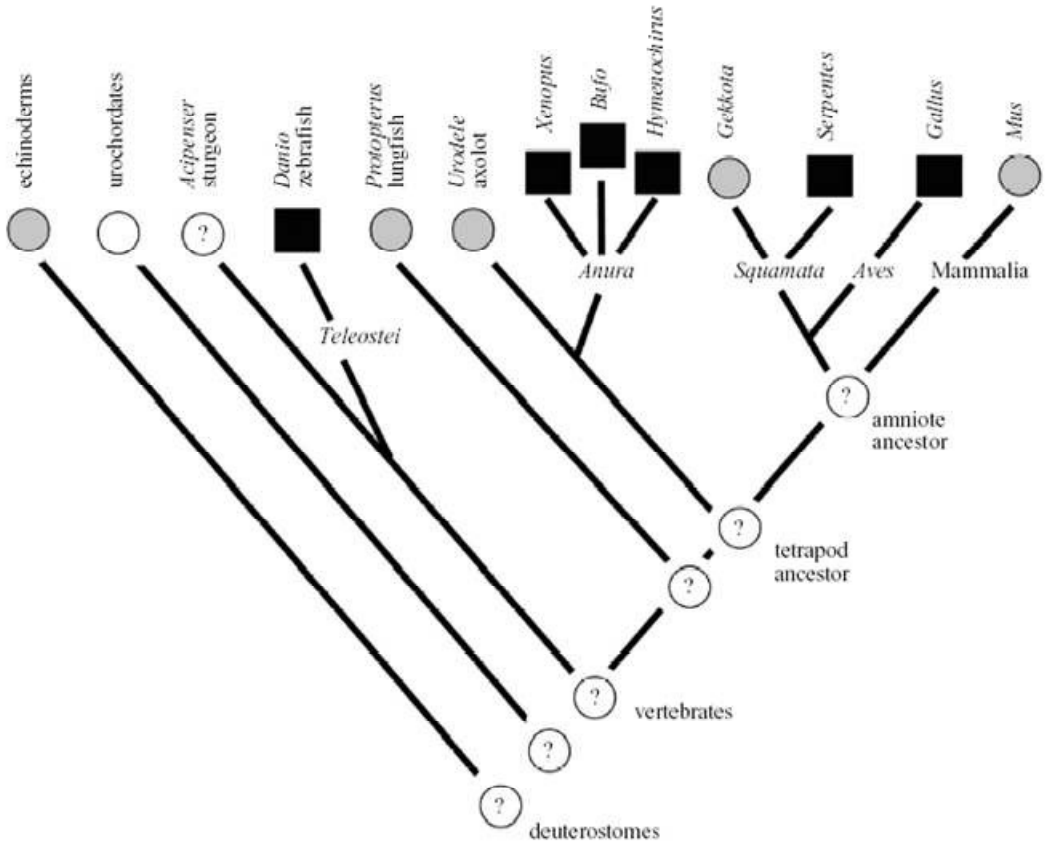


Figure 17. The distribution of germ plasm in animals with either primitive or derived modes of gastrulation. Gastrulation movements are defined as either being primitive to the vertebrate lineage, or a derived mode, as judged by the position of the notochord at the completion of gastrulation (see Johnson *et al.* (2003) for details). Embryos with derived movements tend to give rise to adults with derived adult features. Gastrulation movements are correlated with whether or not available evidence indicates that the embryos in question contain germ plasm, as indicated. Key: circles, conserved embryology; squares, derived embryology; black symbols, evidence for predetermined germ cells; grey circles, evidence for regulative germ cells; open symbols, evidence for mechanism of germ cell determination is inconclusive.

A

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   1 GGCTTGGCCA CGCGTCGACT AGTACGGGGG GGGGGGGGGG GTAGTGGATT TGTTTGTAAG
  61 GTGAGAATCC TTTGAGCGTA CAGATCTTGG ACAAATATGG GTGACGAGAA CTGGGAAGAA
 121 GAACTGGAAT CAACCAGCAC TACTCCATTA GTTCAGAGTT TCAAGAATTT GGATCTCAAG
 181 GATGAAGAGT TTAGCAGTGA TGAGACTACA GGCTCACAAG GTAAGATGTT TGGAGGAGGA
 241 ACAAACAAAT TTGGAAGAGG AAGTATTGGT GATAGTGCAT TTGAAGGATC TGTTTCTTAC
 301 AATGGAAACG TGCCTGACAG GGACCAGTCA GGAAGCAGAA GAGGATTTGG TACTTTTGGC
 361 AGAACTGAAG AATTTGGCAA CGATAGCAAC AGACGAGGAA GAGATGGTGC TGGACCAAGA
 421 CGTGGAAATA TCAGGGGAGG TTTTGGTCAA AGACATGGGG GGATTTGGTG ACACTCATGA
 481 GGAAGTAAGC AGAGATAACT CTGGACCTAG ACGTGGCAGC ACAAGAGGAG GGTTTGTTCG
 541 AAGAGAGGGA GGATTTGGTG ACATTCCAGC GAAGAATAGT AAGGGAATGG ATGATCCTGG
 601 ATTTAGACAT GATACTACAA GAGGAGGATT AAGCCGGAGA GAGGGAGGTG GTTATAAAGG
 661 GAAGGATGAA GAAATTATGT CTGGCTTCAG TACAGAGAAA GCTCCATGGA AAACTGGTAA
 721 AGAAAAAGAT GAAGATCCTG AAGTTCGTAG ATCAGCATAT ATACCACCTC CTCCACCTGA
 781 AGATGAGGAT GCTATTTTTG CACACTATCA GACTGGTATC AACTTCAACA AATATGATGA
 841 AATTCTTGTG AATGTCACTG GACAAGGTGT TCCTCCAGCA GTTCTGACCT TTGAGGAAGC
 901 TGGTTTTAAT GATACGCTAA ATAAGAATAT TGCTAAAACA GGATATGCAA AACCTACTCC
 961 TGTACAGAAG CATGCAATTC CTATTATACT TTCTGGGCGT GATTTAATGG CCTGTGCGCA
1021 GACTGGATCT GGCAAAACTG CAGCTTTTCT GCTACCAATT TTAGCTCAAA TGATGGTGGA
1081 TGGAGTGTCA GCAAGCAGGT TTCAAGAACT GCAAGAACCT GAAGCTATAG TTATTGCTCC
1141 AACCCGGGAA TTAATTTGCC AGATCTATTT AGAAGCAAGA AAGTTTGCTT ATGGAACCTG
1201 TGTAAGGTCT GTTGTTGTCT ATGGCGGAAC AAATACTGGT TATTCAATTC GGCAGATATC
1261 TCAGGGCTGC AACATATTAT GCGCAACTCC TGGAAGGCTC CATGATGTTA TTGGAAAGGG
1321 AAGGATTGGA TTGTCAAAGG TCAGATACCT AGTCCTGGAT GAAGCTGACC GAATGTTGGA
1381 GATGGGATTC ATACCAGATG TTAGGAAGTT GGTGACCTCT CCTGGAATGC CAGCTAAGGA
1441 GGAGCGTCAA ACTTTAATGT TTAGTGCTAC ATTTCCTGAA GAAATTCAGA GGTTGGCTGC
1501 AGAATTTTTG AGAAAGGATT ATTTATTTGT TGCTGTTGGT CAAGTTGGTG GAGCCTGTAG
1561 TGATGTGCAT CAAACTGTTT TAAATGTACC GTATAAATCA AAGCGGAGCA AGTTACATGA
1621 AATCCTTCAG GCTATAGGTT CAGAACGTAC CATGGTATTT GTTGCAACAA AGAAACAAGC
1681 AGATTTCATT GCTACTTATC TTTGTCAAGT AAATCTTCCA ACTACAAGCA TTCACGGTGA
1741 CAGGGAACAG AGAGAGAGGG AAGAAGCTCT AAGAGATTTT CGTTCAGGAA AGTGTCCCAT
1801 TTTGGTTGCT ACATCAGTGG CAGCAAGAGG ACTGGATATC GAAAATGTAC AGCATGTTAT
1861 TAATTTTGAC CTTCCTGAAG GAATAGATGA ATATGTTCAT CGAATTGGTC GAACAGGACG
1921 TTGTGGAAAT GTGGGCAAGG CCATTTCATT CTTCGATCCT ACCAAACCTC AGGATGTTAG
1981 TATTGCTCAG AGTCTTGTGA AAGTGCTTGC TGATGTAAGT TTTTGTAAAT CTTTATAAAA
2041 CTATCGTGCT GAATGGCCTA AATATATGTA ATCTGCATAA TACTAAAAAA AAAAAAAAAA
2101 AAAAAAAA
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B

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   1 MGDENWEEEL ESTSTTPLVQ SFKNLDLKDE EFSSDETTGS QGKMFGGGTN KFGRGSIGDS
  61 AFEGSVSYNG NVPDRDQSGS RRGFGTFGRT EDLATIAPDE EEMVLDQDVE ISGEVLVKDM
 121 GGFGDTHEEV SRDNSGPRRG STRGGFVRRE GGFGDIPAKN SKGMDDPGFR HDTTRGGLSR
 181 REGGGYKGKD EEIMSGFSTE KAPWKTGKEK DEDPEVRRSA YIPPPPPEDE DAIFAHYQTG
 241 INFNKYDEIL VNVTGQGVPP AVLTFEEAGF NDTLNKNIAK TGYAKPTPVQ KHAIPIILSG
 301 RDLMACAQTG SGKTAAFLLP ILAQMMVDGV SASRFQELQE PEAIVIAPTR ELICQIYLEA
 361 RKFAYGTCVR SVVVYGGTNT GYSIRQISQG CNILCATPGR LHDVIGKGRI GLSKVRYLVL
 421 DEADRMLEMG FIPDVRKLVT SPGMPAKEER QTLMFSATFP EEIQRLAAEF LRKDYLFVAV
 481 GQVGGACSDV HQTVLNVPYK SKRSKLHEIL QAIGSERTMV FVATKKQADF IATYLCQVNL
 541 PTTSIHGDRE QREREALRDF RSGKCPILVA TSVAARGLDI ENVQHVINFD LPEGIDEYVH
 601 RIGRTGRCGN VGKAISFFDP TKPQDVSIAQ SLVKVLADVS FCKSL
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C

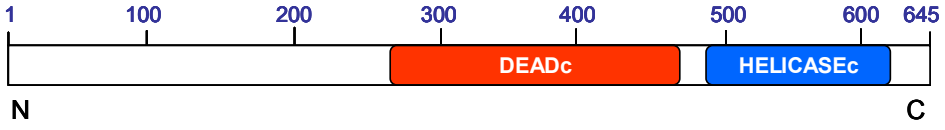

Figure 18. Sequence of *Provh*. *Provh* is a 2124 base pair transcript that codes for a 645 amino acid protein. A. cDNA sequence of *Provh*, with corresponding nucleotide position. B. Predicted amino acid sequence of Provh with corresponding amino acid postion. C. Provh predicted protein diagram illustrating the DEAD box and Helicase domains. N – amino-terminus region, C- carboxy-terminus region

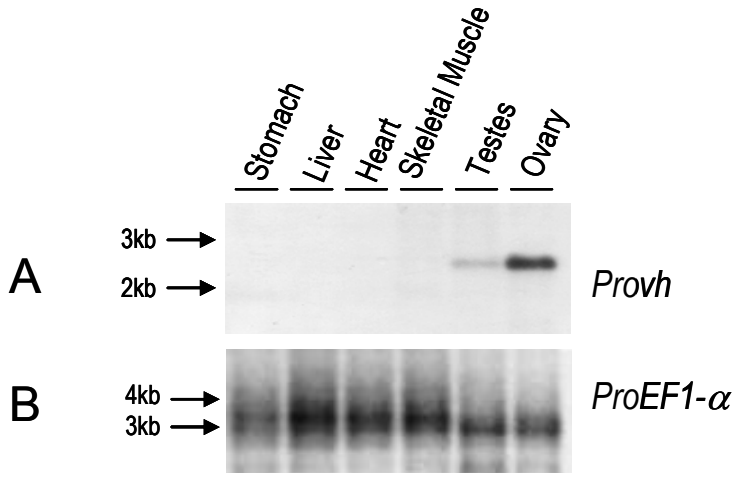

Figure 19. A. *Provh* is restricted to gonadally derived tissue. 10µg of total RNA from the indicated adult lungfish tissues was Northern blotted on to a nylon membrane. The membrane was hybridized with a *Provh* ribonucleotide probe and is indicated by a 2.6kb band. B. RNA integrity was confirmed by stripping and re-probing with *ProEF-1α* is indicated by the 3.5kb band.

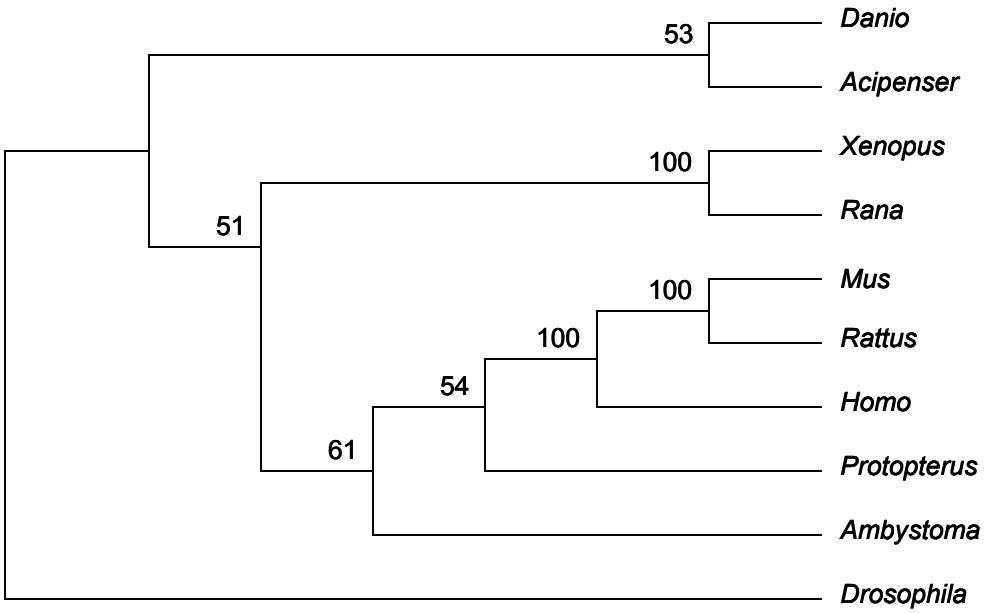

Figure 20. Cladistic analysis of the coding region of vertebrate *vasa* homologs. Drosophila was selected a outgroup to root the tree. Bootstrap values (1000 replicates) with a 50% majority rule, are above branches. Maximum parsimony, and PROTOPARS stepwise matrix was conducted using the EXHAUSTIVE option in PAUP, and analysis yielded a single most parsimonious tree. Ambystoma, Acipenser and Protopterus vasa homologs were cloned at FSU, other sequences were obtained through Genbank see table 8.

CHAPTER 5

METHODS

Degenerate Polymerase Chain Reaction (degPCR)

Polymerase chain reactions using degenerate primers contained the following components: 5µl first strand cDNA, 5µl 10X PCR Buffer (0.5M KCl, 0.1M Tris pH 8.8, 1% Triton X-100, 0.5mM – 3.0mM $MgCl_2$, see Table 4 for $MgCl_2$ concentration for each set of specific primers), 0.4mM dNTP mix, 500ng forward primer, 500ng reverse primer (Primers are listed on table 2 and 0.5 units of recombinant *Taq* polymerase (GibcoBRL). The following parameters listed on table 3 were used to amplify each gene. Ten microliters of each reaction was electrophoresed through a 1.2% agarose gel (GibcoBRL) containing 0.5ug/ml EtBr in 1X TBE buffer at 120 volts for 90 minutes. A molecular weight marker (100bp ladder – New England BioLabs), was run alongside samples to verify the size of the amplified PCR product. PCR products were visualized on a UV light source.

Table 2. Nucleotide sequence of primers used for Degenerate PCR

Primer	Sequence (5′ → 3′)
Axdazl deg Reverse	GC(C/T) TGi AC(A/G) T(A/G)(C/T)TGi GTi A(T/C)i GG
Axdazl deg Forward	GGi GTi (A/T)(G/C)i AA(G/A) GGi TA(T/C) GGi TT
Axvh deg Reverse	CG(T/A) TCi AC(A/G) T(A/G)(T/C) TGi A(G/C)(T/A) AG
Axvh deg forward	CTi T(A/G)(T/C) TGi A(G/C)(T/A) (A/G)Gi (T/C)AC

* i = deoxyinosine

Titering of cDNA libraries

The titer of cDNA libraries was determined by 10-fold serial dilutions from stock tubes. 10µl of stock phage was added to 90µl of SM buffer (0.1M NaCl, 0.08M $MgSO_4 \cdot 7H_20$, 0.05M Tris, pH 7.5, 0.01% Gelatin) then mixed constituting a 10^{-1} dilution.

10µl of the 10^{-1} dilution was added to 90µl of SM buffer then mixed constituting a 10^{-2} dilution. This process was repeated until a 10^{-7} dilution was achieved. 10µl of each phage

Table 3. Thermocycler touchdown reaction conditions for degenerate PCR

Clone	Melt	Anneal	Extension	Cycles
Axvh	10minutes 94°C			1
	1 minute 94°C	1 minute 62°C	1 minute 72°C	3
	1 minute 94°C	1 minute 59°C	1 minute 72°C	3
	1 minute 94°C	1 minute 56°C	1 minute 72°C	3
	1 minute 94°C	1 minute 53°C	1 minute 72°C	3
	1 minute 94°C	1 minute 49°C	1 minute 72°C	3
	1 minute 94°C	1 minute 46°C	1 minute 72°C	3
	1 minute 94°C	1 minute 43°C	1 minute 72°C	3
	1 minute 94°C	1 minute 40°C	1 minute 72°C	30
			10 minutes 72°C	1
Provh	10minutes 94°C			1
	1 minute 94°C	1 minute 62°C	1 minute 72°C	3
	1 minute 94°C	1 minute 59°C	1 minute 72°C	3
	1 minute 94°C	1 minute 56°C	1 minute 72°C	3
	1 minute 94°C	1 minute 53°C	1 minute 72°C	3
	1 minute 94°C	1 minute 49°C	1 minute 72°C	3
	1 minute 94°C	1 minute 46°C	1 minute 72°C	3
	1 minute 94°C	1 minute 43°C	1 minute 72°C	3
	1 minute 94°C	1 minute 40°C	1 minute 72°C	30
			10 minutes 72°C	1
Axdazl	10minutes 94°C			1
	1 minute 94°C	1 minute 68°C	1 minute 72°C	3
	1 minute 94°C	1 minute 65°C	1 minute 72°C	3
	1 minute 94°C	1 minute 62°C	1 minute 72°C	3
	1 minute 94°C	1 minute 59°C	1 minute 72°C	3
	1 minute 94°C	1 minute 56°C	1 minute 72°C	3
	1 minute 94°C	1 minute 53°C	1 minute 72°C	3
	1 minute 94°C	1 minute 49°C	1 minute 72°C	30
			10 minutes 72°C	1

dilution was added to 300µl of the appropriate host strain of bacteria (Y1090) or (LE392). Optical density (OD) of the host was between 0.3 and 0.6 or during the log phase of growth. The phage/cell culture was mixed and incubated at 37°C for 15 minutes. Five milliliters (ml) of NZCYM (10g/L NZ amine, 0.085M NaCl, 5g/L bacto-yeast extract, 1g/L casamino acids, 0.008M $MgSO_4 \cdot 7H_20$, 0.7% agarose) was heated to 55°C and then added to culture. The

solution was then mixed and then spread on 100mm Petri dish containing Luria-Bertani Agar (LB, 0.17M NaCl, 10g/L bacto-tryptone, 5g/L bacto-yeast extract plus 20g/L Bactoagar (Difco). Plates were allowed to solidify at 22°C, than incubated at 37°C for 15-18 hours. Only plates that contained between 20 and 200 plaque forming units (pfu's) were counted. The titer was determined by multiplying the number of plaque forming units (pfu's) and the dilution factor together.

Lifting of cDNA libraries

Nylon membrane filters (Osmonics) were placed over plated phage (in duplicate) and allowed to sit for one minute. Holes were punched in the membranes with a 18 gauge needle to determine orientation later then removed with a pair of forceps and placed upside down on a piece of Whatmann 3MM (Fisher) paper to dry. After drying, membranes were placed "phage side up" on Whatmann 3MM paper soaked with denature solution (0.5M NaOH, 1.5M NaCl) for five minutes. Membranes were then transferred to Whatmann 3MM paper soaked with neutralization solution (0.5M Tris pH 7.5, 1.5M NaCl) for five minutes. Membranes were then removed and placed on Whatmann 3MM paper to dry then placed in a UV cross-linker (BioRad) and cross-linked at 50 milli-Joules. Membranes were wrapped in plastic wrap and stored at -20°C until use.

Prehybridization of membranes

Membranes were rinsed in 2X SSC (0.3M NaCl, 0.03M $Na_3C_6H_5O_7 \cdot 2H_20$) to remove dried salts. Membranes were then placed in four separate hybridiation bags. Duplicate membranes were not placed in the same bags. 25ml of prehybridization solution (1X High Phosphate Buffer (0.5M NaCl, 0.1M $Na_2HPO_4 \cdot 7H_20$, 0.005M $EDTA \cdot 2H_20$), 1% Sarkosyl, 200µg/ml denatured salmon sperm DNA) was added to each bag and allowed to prehybridize at 65°C for a minimum of four hours.

Radioactive labeling of DNA probes

50ng of DNA was added to the following reaction: 2.5µl random hexamers, 6.5µl H_2O and then boiled for five minutes at 100°C and placed at 37°C. Then the following was added to the boiled DNA: 2µl 10X Random priming buffer (900mM Hepes pH 7.4, 100mM

$MgCl_2$), 2µl dithiothreital (DTT), 2µl 0.5mM dNTP's (minus dATP), 2µl dαATP labeled P^{32}, 1µl H_2O, and 1µl Klenow enzyme (New England BioLabs). The reaction was allowed to proceed for one hour at 37°C. The radiolabeled DNA was precipitated by addition of the following: 30µl H_2O, 50µl 5M NaoAC, 10µl 1mg/ml muscle glycogen (Roche) and 275µl 100% ethanol. This was then mixed and placed at –20°C for 20 minutes. The DNA was centrifuged for 5 minutes and then the supernatant was removed, the pellet air dried and resuspended in 100µl H_2O. The resuspended DNA was boiled at 100°C for five minutes and then added to the prehybridization solution. Hybridization was allowed to proceed for 16-18 hours at 65°C. Note for library screening: four separate reactions were labeled using 5 µl of α-dATP-P^{32} each. One reaction was placed in each bag for hybridization.

Washing of radioactive membranes

Library membranes were washed from "low" to "high" stringency. Hybridization solution was removed and radioactive membranes were placed in a solution of 2X SSC, 0.1% SDS on a rocker device for 20 minutes at room temperature (RT). The stringency of washes was increased as follows: 1X SSC, 0.1% SDS at RT, 0.5X SSC, 0.1% SDS at RT, 0.1X SSC, 0.1% SDS at RT each for 20 minutes.

Purification of primary pfu's to single phage

A dilution between 10^{-2} and 10^{-5} of each primary plug was plated on LB/agarose plates as previously stated (see titering of cDNA libraries). Plates that contained between 1000-2000 pfu's were used (secondary plates). Secondary plates were lifted, prehybridized, hybridized, and washed as previously stated. Secondary membranes were exposed overnight at -70°C. If single phage were not obtained from secondary plates, a third round of screening was preformed as previously stated. Homogeneous phage display complete reactivity with the radioactively labeled probe. Single phage were cored with the small end of a Pasteur glass pipette, placed in 100µl of SM buffer along with 10µl of chloroform and eluted overnight at 4°C.

Preparation of Plate Stocks

5µl of each single phage was plated on small LB/agarose plates as previously stated and grown overnight at 37°C. Three milliliters of SM buffer was added to each plate and then placed on a shaker at room temperature for no less than 3 hours to elute phage. SM buffer containing eluted phage was removed and placed in 1.5ml eppendorf tubes to which 100µl of chloroform was added. Phage stocks were stored at 4°C.

Preparation of lambda DNA (Lambda Lysis Preps)

DH10b *E. coli* cells were grown to mid-log phase (O. D. = 0.5) at 37°C. 300µl of the host strain was infected with 1-2µl of phage from a plate stock and incubated for 15 minutes at 37°C. 10ml of NZCYM broth was added to the infected cells and allowed to nourish the phage at 37°C for 5-7 hours. When lysis was complete, 1ml of chloroform was added. 7ml of the lysate was then transferred to a 15ml Falcon screw cap tube (Fisher). 7µl of DNase (Sigma) (10mg/ml) and 10µl of RNase (Sigma) (10mg/ml) were added to the lysate, mixed and incubated at 37°C for 30 minutes. The lysates were then placed in a tabletop centrifuge and rotated at 3,750 rpm for 30 minutes to pellet remaining bacteria cells. The supernatant was removed and placed in a clean 1.5 ml tube. 2ml of STE buffer (1.5% SDS, 0.3M Tris pH 9.0, 0.15M EDTA) was added to the supernatant, mixed and incubated at 70°C for 15 minutes. Tubes were allowed to cool to room temperature for 10 minutes and then 1.5 ml of 8M KoAc was added, mixed and placed on ice for no less than 30 minutes. Tubes were then centrifuged at 3,750 rpm for 30 minutes to pellet proteins. Supernatants were then filtered through a Kimwipe (Kiberly Clarke) into a 30ml glass Corex tube to which 0.6 volumes of Isopropanol was added and allowed to precipitate at room temperature for 30 minutes. Lambda DNA was pelleted in a Sorvall centrifuge at 10,000 rpm and the supernatant was discarded. The pelleted DNA was washed with 5ml of 70% ethanol and then centrifuged at 10,000 rpm for 10 minutes. Supernatants were discarded and pellets were allowed to air dry at room temperature. Lambda DNA was resuspended in 500µl TE (10mM Tris pH 8.0, 1mM EDTA pH 8.0) and then transferred to a 1.5ml microfuge tube. Lambda DNA was extracted with 500µl phenol/chloroform (1:1) and centrifuged for 5 minutes. Supernatants were removed, and 50µl 5M NH_4oAC and 1ml 100% ethanol was added and placed at –20°C for

20 minutes. Tubes were centrifuged for 5 minutes, supernatants removed and pelleted lambda DNA was allowed to air dry at room temperature. TE was added to pellet until DNA was resuspended. Quantification of lambda DNA was done by gel electrophoresis containing 0.5ug/ml ethidium bromide.

Restriction Digest of Lambda DNA

25µg of lambda DNA containing cDNA was restriction digested in a 360µl reaction containing: 1X React 3 buffer (GibcoBRL), 10mM DTT, 6mM spermidine and 125 units of EcoRI enzyme (GibcoBRL). Reactions were allowed to incubate at 37°C for no less than 2 hours. After digestion, the reaction was extracted with 360µl phenol/chloroform, precipitated with 40µl 5M NH_4oAC and 1ml 100% ethanol and placed at –20°C for 20 minutes. Tubes were centrifuged for 5 minutes, supernatants removed and pelleted lambda DNA was allowed to air dry at room temperature. Lambda DNA was resuspended in 100µl TE.

Purification of digested cDNA by low melting point (LMP) gel electrophoresis

Digested lambda DNA was electrophoresed through a 1.2% LMP agarose gel (GibcoBRL) containing 0.5ug/ml EtBr in 1X TAE (0.04M Tris acetate, 0.001M EDTA) buffer at 75 volts until bromophenol blue running dye was three-quarters through the gel. Molecular weight markers (100kb ladder and 100bp ladder – New England BioLabs) were run alongside samples. Digested cDNAs were visualized on a UV light source, cut from the agarose gel with a razor and placed in a 1.5ml eppendorf tube.

Extraction of cDNA from LMP agarose gel

For every 100mg of gel, 180µl of H_20 and 20µl of 10X β-agarase buffer (New England BioLabs) was added to each gel slice. Gel slices were then placed at 70°C until melted. Tubes were cooled to 40°C then 1 unit/100mg gel slice of β-agarase enzyme was added, mixed and incubated at 40°C for 1 hour. The reactions were then extracted twice with a 1:1 ratio of phenol. The reaction was precipitated with 1/10 volume 5M NH_4oAC, 10ug muscle glycogen (Boeringher) and 2.5X 100% ethanol and placed at –20°C for 20 minutes.

Tubes were centrifuged for 5 minutes, supernatants removed and the cDNA was allowed to air dry at room temperature. Pelleted cDNA was resuspended in 20µl TE. Recovery and quantification of cDNA was verified by electrophoresing 1µl in a 1.2% agarose gel containing 0.5ug/ml ethidium bromide in 1X TAE buffer.

Preparation of plasmid vector for ligation

A restriction digest containing 10µg of pBluescript SK (Stratagene) plasmid, 1X React 3 buffer (GibcoBRL), 10mM DTT, and 50 units of *Eco*RI enzyme (GibcoBRL). Reactions were allowed to incubate at 37°C 1 hour. The plasmid vector was dephosphorylated by addition of the following: 1/10 volume 10X dephosphorylation buffer (GibcoBRL) and 0.1 units Calf Intestinal Phophatase (CIP) (New England BioLabs). Reaction was incubated at 37°C for 30 minutes. After dephosphorylation, the reaction was extracted with phenol/chloroform, precipitated with 1/10 volume 5M NH_4oAC and 2.5X 100% ethanol and placed at –20°C for 20 minutes. Tubes were centrifuged for 5 minutes, supernatants removed and pelleted DNA was allowed to air dry at room temperature. DNA was resuspended in 100µl TE. Estimation of the quantity of plasmid vector DNA was verified by electrophoresing 1µl in a 1.2% agarose gel containing 0.5ug/ml EtBr in 1X TAE buffer.

Ligation of cDNA into plasmid vector

A 20µl ligation reaction was performed with the following: 100ng cDNA containing *Eco*RI ends, 25ng pBluescript SK (Stratagene) plasmid digested with *Eco*RI, 1X T4 ligase buffer (containing 1mM ATP) and 1 unit T4 DNA ligase enzyme (New England BioLabs). Ligation was incubated overnight at room temperature and then placed at 70°C for 5 minutes to inactivate ligase.

Transformation of ligated cDNA into bacteria

Frozen chemically competent bacteria cells (DH5α or DH10B) at $1X10^7$ colony forming units (cfu's) per microgram of DNA were used in all transformations. 5µl of each ligation was added to 200µl of competent cell and placed on ice for 10 minutes. Tubes were

placed at 42°C for one minute to "heat shock" bacteria and then placed back on ice. Transformed cells were spread on LB/agarose plates containing 100ug/ml ampicillin (Fisher) and 200ug/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, GibcoBRL). Plates were allowed to dry and then placed at 37°C for 16-18 hours.

Plasmid minipreps

1.5ml of each culture was transferred to 1.5 ml microfuge tubes. Tubes were centrifuged for 30 seconds to pellet bacterial cells. Supernatants were then removed. 100µl of Solution I (10mM Tris pH 8.0, 1mM EDTA pH 8.0, 20% sucrose (w/v)) was added to each tube and then votexed to resuspend bacteria. 100µl of Solution II (0.2M NaOH, 1% SDS) was added to each tube and then gently mixed to lyse bacteria. 100µl of Solution III (3M NaoAc pH 4.6) was added to each tube then gently mixed until a white precipitate was seen. Tubes were centrifuged for 5 minutes to pellet precipitate. Precipitate was then removed with an autoclaved toothpick and 300µl of phenol/chloroform (1:1) was added and mixed. Tubes were centrifuged for 5 minutes and then the aqueous layer was removed and placed in another tube. One hundred and eighty microliters of 100% isopropanol was added to each tube to precipitate plasmid DNA. Tubes were placed at -20°C for 20 minutes. Tubes were centrifuged for 5 minutes supernatants removed and pelleted DNA was allowed to air dry at room temperature. DNA was resuspended in 50µl TE containing 0.5µl RNase Cocktail (Ambion) and placed at 37°C for 20 minutes.

Preparation and sequencing of plasmid DNA

Plasmid DNA used for sequencing was prepared by using a plasmid DNA miniprep kit from Qiagen. Additionally, the plasmid DNA was precipitated with 1/10 volume 5M NH_4oAC and 2.5X 100% ethanol and placed at –20°C for 20 minutes. Tubes were centrifuged for 5 minutes and supernatants removed. Pelleted DNA was washed twice with 70% ethanol and allowed to air dry at room temperature. DNA was resuspended in 30µl H_2O. Quantification of plasmid DNA was determined by absorption at 260nm in a DU-Beckman spectrophotometer. All sequencing was performed by the DNA Sequencing Facility at Florida State University using the universal primers, T7 and T3 or Abridged

Universal Amplification Primer (AUAP) (GibcoBRL) see table 4. Sequences were converted to electronic files for analysis.

Table 4. Nucleotide sequence of primers used for DNA sequencing

Primer	Sequence (5′ → 3′)
AUAP	GGCCACGCGTCGACTAGTAC
T3	TAACCCTCACTAAAGGGA
T7	TAATACGACTCACTATAGGG

Analysis of cDNA sequences

Assembly and translation of sequences were done by using DNA Strider 1.2. Homology searching was performed using the BLAST program at NCBI (Altschul et al., 1990). Amino acid alignments were performed by a combination of comparison to the Swissprot database and ClustalW programs (http://www2.ebi.ac.uk/clustalw/).

5′ RACE (Rapid Amplification of cDNA Ends) of Axvh and Axdazl

Total RNA from testes was extracted using Trizol (GibcoBRL) according to the manufacturer's instructions. Total RNA was resuspended on ice by the addition of H_2O until the pellet was solubilized. Quantification of RNA was determined by absorption at 260nm in a DU-Beckman spectrophotometer. Integrity of total RNA was determined by gel electrophoresis. Total RNA was used in the 5′ RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (GibcoBRL) according to the manufacturer's instructions Primers used for *Axvh* and *Axdazl* are summarized in table 5.

Table 5. Nucleotide sequence of primers used for 5′ RACE of *Axdazl and Axvh*

Primer	Sequence (5′ → 3′)
AAP	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG*
Axdazl 5′ Reverse	CGCTATCATACCGATCGATCC
Axdazl 5′ Reverse nested	CGTAATAGCCACTGTTGCCGG
Axdazl PCR Reverse	GATTACCATGAATCATCCTC
Axvh 5′ Reverse	CCCGATCTCCGCTTCACTCG
Axvh 5′ Reverse nested	GGGCATTAATCAGTCAGAGGC
Axvh PCR Reverse	CATTAACAATGCCCGGCATCG

* I = deoxyinosine

Tissue Dissection/Embryo staging and dissection

Adult animals were given a lethal dose of tricaine mehane sulphonate (MS222, tissues were dissected using sterile instruments and then frozen on dry ice in a 50ml blue screw cap tube (Fisher). All axolotl embryo staging was according to Bordzilovskaya *et al.,* (1989). Embryos were either obtained from spawnings from the colony at Florida State University or from the Axolotl Colony at Indiana University. Embryos were cultured in their jelly coats until collection in 20% Holtfreter's Solution (0.3mM NaCl, 0.03mM KCl, 0.045mM $CaCl_2$, 0.01mM $NaHCO_3$). Embryos were manually dejellied and dissected in 20% Holtfreter's Solution. Whole embryos and dissected parts were collected and frozen on dry ice in a minimal amount of media.

Collection of amphibian oocytes

Mature *A. mexicanum* were sacrificed by rapid decapitation and parts of their ovaries were removed and placed in normal 20 %Holtfreter's Solution. Large female *X. laevis* were immersed in 0.1% (w/v) aqueous solution of MS 222. Ovarietomies were performed and dismembered tissue was placed in normal 20% Holtfreter's Solution. In both cases, the ovary was dissected and oocytes were staged according to Dumont (1972). Stage 1 and 2 oocytes were washed in 0.1M $NaPO_4$ pH-7.5 and follicular cells were removed using a 0.1% (w/v) collagenase in 0.1M $NaPO_4$.

Florescence and confocal studies

Individual *A. mexicanum* and *X. laevis* oocytes were incubated in 500nM MitoTracker Red (Molecular Probes) dissolved in OR2 (82.5mM NaCl, 2.5mM KCl, 1.0mM $CaCl_2$, 1.0mM $MgCl_2$, 1.0mM Na_2HPO_4, 5.0mM HEPES) for 0.5h at 27°C. Labeled oocytes were washed with PBS and fixed in MEMFA. Post fixation washes were done using PBS and whole oocytes were examined using a Zeiss 1045 quantitative florescent microscope. Confocal images were obtained with a Zeiss LSM 410 laser scanning microscope. All images were acquired using a 25x oil immersion objective and z-scan intervals were at 3.5μm. All Florescence microscopy was done with the help of Lynnette Gerhold at the Flordia State University microscopy lab.

Total RNA extraction

Total RNA was extracted by two methods. For extraction of total RNA from adult tissues the Lithium Chloride/Urea method was utilized. Five volumes of 3M LiCl/6M Urea was added to each frozen tissue and then homogenized with a Polytron until no whole tissue was visible. Tubes were then placed on ice at 4°C overnight to allow precipitation. Tubes were placed in a tabletop centrifuge and spun at 3,750 rpm for 20 minutes to pellet RNA. Supernatants were discarded and two volumes of TE/1% SDS were added to each sample vortexed than resuspend RNA. Samples were extracted with an equal volume of phenol/chloroform (1:1) until the interface was clear. One-tenth the volume of 5M NH_4oAC and 2.5X 100% ethanol was added to each tube and placed at –20°C for 20 minutes. Tubes were centrifuged for 10 minutes, supernatants removed and pelleted RNA was allowed to air dry at room temperature. RNA pellets were resuspended in 360µl H_2O and then transferred to a 1.5ml eppendorf tube. Total RNA was re-precipitated by the addition of 40µl 5M NH_4oAC and 1ml 100% ethanol was added to each tube and placed at –20°C for 20 minutes. Tubes were centrifuged for 5 minutes, supernatants removed and pelleted RNA was allowed to air dry at room temperature. Total RNA was resuspended on ice by the addition of H_2O until pellets were solubilized. Quantification of RNA was determined by absorption at 260nm in a DU-Beckman spectrophotometer. Integrity of total RNA was determined by gel electrophoresis.

For extraction of total RNA from staged embryos, or dissected embryo parts, Trizol (GibcoBRL) was used according to the manufacturer's instructions. Total RNA was re-precipitated by the addition of equal volume of 8M LiCl and subsequent incubation at 4°C overnight. Tubes were centrifuged for 5 minutes and supernatants removed. Pelleted RNA was washed twice with 70% ethanol and allowed to air dry at room temperature. Total RNA was resuspended on ice by the addition of 10µl H_2O per embryo collected. Quantification of RNA was determined by absorption at 260nm in a DU-Beckman spectrophotometer. Integrity of total RNA was determined by gel electrophoresis.

Northern Blotting

3 volumes of RNA loading buffer (66% Formamide, 13% 10X MOPS, 20% formaldyhyde) was added to 5μg of adult RNA or one-half embryo equivalents. Samples were boiled at 100°C for 3 minutes then placed at 70°C. One microliter of 1mg/ml EtBr and running dye were added to each sample. Total RNA was electrophoresed in a 1.2% agarose gel in 1X MOPS buffer (0.02M MOPS pH 7.4, 0.008 NaoAc, 0.001M EDTA) containing formaldehyde at 85 volts until bromophenol blue running dye was three-quarters through the gel. A molecular weight marker (RNA ladder – GibcoBRL) was run alongside samples. Total RNA was visualized on a UV light source. After electrophoresis, formaldehyde gels were then placed on a piece of Whatmann 3MM paper on a sponge soaked in 20XSSC. A piece of nylon membrane (Osmonics) was cut to the size of the gel, hydrated in H_2O, soaked in 20XSSC and placed on top of the gel. 2 pieces of Whatmann 3MM paper were soaked in 20XSSC and then placed on top of the membrane. A stack of paper towels cut to the size of the gel was placed on top of the two pieces of Whatmann 3MM paper. A glass plate was placed on top of the paper towels along with a weight. The total RNA was allowed to transfer for 14-16 hours. After blotting was complete, the membrane was placed on Whatmann 3MM paper to dry, then placed in a UV cross-linker (BioRad) and cross-linked at 50 milliJoules. Membranes were wrapped in plastic wrap and stored at -20°C until use.

In vitro synthesis of radioactive antisense RNA

The orientation of cloned cDNAs within recombinant plasmids was determined by sequencing. 5μg of cloned cDNAs were digested with a single restriction enzyme to generate a linear template. Five hundred nanograms of digested plasmid and undigested plasmid were electrophoresed and visualized by EtBr staining to verify complete digestion of template. Reactions were phenol/chloroform extracted, precipitated, dried and resuspended in TE at a concentration of 500mg/ml. An *in vitro* transcription reaction contained the following components: 500ng linearized template, 1X transcription buffer (New England BioLabs), 0.5mM NTP mix, 20μCi αUTP-P^{32}, 20 units RNase inhibitor (Amersham), and 50 units T7 or T3 RNA polymerase (New England BioLabs). Reactions were incubated at 37°C for 1 hour. After incubation, 30μl of STE buffer (0.1M NaCl, 0.01M Tris pH 8.0, 0.001M EDTA) was added and then purified from a ProbeQuant G-50 micro spin column

(Amersham) by chromatography to remove unincorporated nucleotides. An equal volume of 100% formamide was added after nucleotide removal.

Detection of RNA on Northern Blots

embranes were rinsed in 2X SSC to remove dried salts; they were then placed in hybridization bags. Northern prehybridization solution (50% formamide, 50mM $NaPO_4$ pH 6.4, 5X SSC, 0.1% SDS, 1mM EDTA, 0.05% Bovine Serum Albumin, 0.05% Ficoll, 0.05% PVP, 200μg/ml denatured salmon sperm DNA) was added to each bag and allowed to prehybridize at 65°C for a minimum of 4 hours. Radioactively labeled anti-sense RNA was used for probe. Probes were boiled at 100°C for 5 minutes to denature RNA then added to hybridization solution. Membranes were hybridized for 14-16 hours at 65°C. Membranes were washed in 0.1X SSC, 0.1% SDS at 65°C for 20 minutes. Radioactive membranes were wrapped in plastic wrap and placed in a cassette with a piece of X-ray film and a intensifying screen. Film was exposed at -70°C for overnight up to 3 days. X-ray film was developed using an automatic developer.

In vitro transcription of digoxygenin labeled antisense RNA probes for sectioned In Situ Hybridization

Digoxygenin labeled antisense RNA probes were generated according to Harland (1991). With the *in vitro* transcription reaction containing the following components: 2.5 μg linearized template, 1X transcription buffer (New England BioLabs), 0.5mM NTP mix containing digoxygenin labeled UTP (Boeringher), 10mM DTT, 20 units RNase inhibitor (Amersham), and 90 units T7 or T3 RNA polymerase (New England BioLabs). Reactions were incubated at 37°C for 2 hours, treated with 1 unit of RQ1 DNase (Promega) for 15 minutes at 37°C. Reactions were then passed through a ProbeQuant G-50 micro spin column (Amersham) to remove unincorporated nucleotides. Quantification of synthetic RNA was determined by absorption at 260nm in a DU-Beckman spectrophotometer assuming 40μg RNA/O. D. 260nm. Integrity of synthetic RNA was determined by gel electrophoresis. Digoxygenin labeled probes were diluted to a concentration of 10μg/ml in hybridization solution and stored at -20°C until use.

Microinjection of Embryos

Fertilized eggs were obtained from spawnings at Florida State University. Fertilized eggs were manually dejellied and then placed on ice until use. Before use fertilized eggs or two-cell embryos were placed in 10% Ficoll in 100% Holtfreter's solution to shrink the peri-vitelline space. 15ng of synthetic BMP-4 and eFGF RNA or equal volume of H_2O was microinjected with a pulled glass needle into one- or two-celled embryos using a Narishige IM 300 microinjector and a Narishige manipulator. Injected embryos were slowly changed from 10% Ficoll/100% Holtfreter's solution to 10% Holtfreter's solution. Embryos were cultured in 10% Holtfreter's solution at 22°C until they reached blastula stage (stage 8; Bordzilovskaya et al., 1989). Animal caps were removed via microdissection and retured to 10% Holtfreter's solution. Animal caps were harvested when sibling embryos reached early larval stage (stage 43).

Sectioned In Situ Hybridization

All sectioned *in-situ* experiments were done in collaboration with Rosemary Bachvarova, Weill school of medicine, Cornell University New York. Sections of axolotl embryos or oocytes were fixed in MEMPFA (0.1 M MOPS pH 7.4, 2mM EGTA, 1mM $MgSO_4$, 4% paraformaldehyde) overnight at room temperature. Fixative was replaced with methanol cooled to -20°C. sections were rehydrated in successive 5 minute washes in: 75% methanol: 25% 1X PBSTw (1X Phosphate Buffered Saline (8g/L NaCl, 0.2g/L KCl, 1.44g/L Na_2HPO_4, 0.24g/L K_2HPO_4), 0.1% Tween 20), 50% methanol: 50% 1X PBSTw: 25% methanol: 75% 1X PBSTw. Sections were then washed in PBSTw three times for 5 minutes each then were rinsed in 2mls of 0.1M Triethanolamine (Sigma) twice for 5 minutes each. 5µl of acetic anhydride was added, mixed gently an additional 5µl of acetic was added, mixed gently and equilibrated for 5 minutes. Sections were then washed twice for 5 minutes in 1X PBSTw and then bleached (Bertwistle et al., 1996) in 2mls of 5% Formamide, 0.5X SSC, 10% H_2O_2 on a light box until pigment was removed. Sections were washed twice for 5 minutes in 1X PBSTw and then refixed in 4% formaldehyde/1X PBSTw for 20 minutes. Sections were washed 5 times for 5 minutes in 1X PBSTw. All of the solution other than approximately 1ml was removed and 250µl of hybridization buffer (50% formamide, 1.3X

SSC pH 5.0 with citric acid, 5mM EDTA, 50μg/ml yeast RNA (Boeringher), 0.2% Tween-20 (Sigma), 0.5% CHAPS (Fisher), 100μg/ml Heparin (Sigma) was added. After 5 minutes, the buffer was replaced with 0.5ml hybridization buffer. The slides were placed at 68°C in a standing H_2O bath. The buffer was then replaced with 1ml of hybridization buffer and prehybridized for at least 6 hours at 68°C. Buffer was then replaced with 1ml of hybridization buffer containing 1μg/ml digoxygenin labeled probe. Probes were boiled for 8 minutes in hybridization buffer at 100°C before adding to embryos and placed at 70°C to cool. Probes were allowed to hybridized overnight at 68°C in standing H_2O bath.

Sections were washed in 2mls in the following solutions: hybridization solution once for 10 minutes at 68°C, 50% hybridization solution/2X SSC/0.3% CHAPS once for 10 minutes at 68°C, 25% hybridization solution/2X SSC/0.3% CHAPS once for 10 minutes at 37°C, and 2X SSC/0.3% CHAPS twice for 10 minutes at 37°C. Slides with sections were then washed in 2X SSC/0.3% CHAPS containing 20mg/ml RNase A (Sigma) and 10 units/ml RNase T1 (Sigma) once for 30 minutes at 37°C. After RNase digestion, sections were washed in 2mls under the following conditions: 2X SSC/0.3% CHAPS once for 10 minutes at room temperature, 0.2X SSC/0.3% CHAPS twice for 30 minutes at 68°C in a standing H_2O bath, 1X PBSTw/0.3% CHAPS twice for 10 minutes at 68°C in standing H_2O bath, and then 1X PBSTw once for 10 minutes at room temperature. Sections were washed in 1X MABT buffer (100mM Maleic acid pH 7.5, 150mM NaCl, 0.1% Tween) twice for 10 min at room temperature. Embryos were then incubated in 2ml of 1X MABT/2% Boeringher Blocking reagent for a minimum of 1 hour or longer. Buffer was replaced with 1ml of 1X MABT/2% Boeringher Blocking Reagent containing 1:2000 dilution of anti-digoxygenin antibody conjugated to alkaline phosphatase (Boeringher). Antibody was allowed to incubate overnight at 4°C. Sections were then washed in 1X MABT six times for 30 minutes each. Embryos were equilibrated in NTMT buffer (100 mM Tris pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 1% Tween-20) twice for 10 minutes at room temperature. Buffer was replaced with 1ml of BM Purple (Boeringher) and placed at 4°C overnight.

Sections were rinsed twice in NTMT and replaced with 1 ml of BM Purple and incubated at room temp in the dark or placed back at 4°C. Signal was monitored closely at

room temp until optimal signal was achieved. Color reactions were stopped by washing 3 times in 1X PBSTw then embryos were fixed in MEMFA.

Reverse Transcription of Total RNA to First-strand cDNA

One microgram of total RNA from adult tissues or from one-half embryo RNA equivalents of staged embryos, or dissected parts, were used in all reverse transcription (RT) reactions. RT reactions contained the following components in addition to total RNA: 1X reverse transcription buffer (New England BioLabs), 1mM dNTP mix, 1µg oligo dT, 20 units RNase inhibitor (Amersham), and 400 units MMuLV reverse transcriptase (New England BioLabs). A negative control reaction contained all components except the reverse transcriptase enzyme. Reactions were incubated at 40°C for 45 minutes. After incubation, 75µl of H_2O was added and placed at 70°C for five minutes to denature enzymes. Reactions were stored at -20°C.

Semi-quantitative RT-PCR

The sequence of primers, magnesium chloride concentrations and annealing temperatures for each set of primers can be found in Table 6. The number of cycles for each gene amplified was determined empirically (see Table 7). PCR reactions were performed and the products were separated by electrophoresis through a 1.2% LMP agarose gel (GibcoBRL) containing 0.5ug/ml EtBr in 1X TAE buffer at 100 volts until bromophenol blue running dye was three-quarters through the gel. PCR products were visualized on a UV light source. If no product was visualized by EtBr staining, the products were considered to be in the linear range of amplification. Gawantka et al., (1995) consider visible products within the linear range. If products were visualized, the reactions were repeated at fewer cycles based on the quantity of product seen by EtBr staining. PCR products were then blotted to nylon membranes and Southern hybridization was performed using probe from the cloned cDNAs as described above. Expression of each gene was visualized by autoradiography.

Table 6. Nucleotide sequence of primers used in analysis of RNA by RT-PCR

Gene	Forward Primer (5´ → 3´)	Reverse Primer (5´ → 3´)	$MgCl_2$ Concentration (10X)	Annealing Temp.
Axdazl	CTTGGACTCTGGTGATGG	AGATTCCATTCCGATGAAGG	2.5mM	51°C
Axvh	TCTGACCGCTGAAGACAAGG	ATGTGCTTGGCGGCTTCACC	2.5mM	50°C
Axvg-1	GCACTCCGACGAAGACACC	CAACCGGCGACAGAAGGAG	2.5mM	56°C
AxEF-1α	GGTGTTGGACAAGCTGAAGG	CGTGCCAGCCAGAGATTGG	3.0mM	57°C

Table 7. Number of PCR cycles performed in each indicated RT-PCR experiment

Gene	Dev. Series	Dissected Parts	Adult Tissues
Axdazl	25	--	--
Axvh	25	--	--
Axvg-1	--	25	--
AxEF-1α	26	--	30

Southern Blotting

All DNA samples were electrophoresed as previously stated. Gels were then placed in a solution of 0.5M NaOH, 1.5M NaCl for 20 minutes then transferred a solution of 0.5M Tris pH 7.5, 1.5M NaCl for another 20 minutes. Gels were then placed on a piece of Whatmann 3MM paper on a sponge soaked in 20XSSC. Nylon membrane (Osmonics) was cut to the size of the gel, hydrated in H_2O, soaked in 20XSSC and then placed on top of the gel. Two pieces of Whatmann 3MM paper were soaked in 20XSSC and then placed on top of the membrane. A stack of paper towels cut to the size of the gel was placed on top of the two pieces of Whatmann 3MM paper. A glass plate was placed on top of the paper towels along with a weight. The DNA was allowed to transfer for 14-16 hours. After blotting was complete, the membrane was placed on Whatmann 3MM paper to dry then placed in a UV cross-linker (BioRad) and cross-linked at 50 milliJoules. Membranes were wrapped in plastic wrap and stored at -20°C until use.

Southern Hybridization

Membranes were rinsed in 2X SSC to remove dried salts. Membranes were then placed in Hybridization bags. Ten milliliters of prehybridization solution (1X High Phosphate Buffer, 1% Sarkosyl, 200μg/ml denatured salmon sperm DNA) was added to each bag and allowed to prehybridize at 65°C for a minimum of 2 hours. DNA was labeled with radioactive nucleotides and hybridized as described above. Membranes were washed in twice in 0.1X SSC, 0.1% SDS at 65°C for 20 minutes. Radioactive membranes were wrapped in plastic wrap and placed in a cassette with a piece of X-ray film and an intensifying screen. Film was exposed at -70°C according to the amount of radioactivity as determined by a Geiger counter. X-ray film was developed using an automatic developer.

CONCLUSIONS

We set out to build the axolotl as a viable model system to study regulative germ cell formation, and use this system to dissect the molecular pathways that lead to the production of PGCs using conventional technologies developed for *Xenopus* embryos. As no other non-mammalian model system produces PGCs by regulative mechanisms, axolotl embryos provide unique advantages towards understanding how maintenance of the totipotent condition is governed during vertebrate development.

Our initial focus was to isolate markers that would enable us to characterize the development of PGCs, related cell types, and the tissues from which they arise. We started by examining the expression of *Axvh* (Chap. 2) and *Axdazl*, (Chap. 3) two genes that are required proper germ cell formation in all organism described to date. We showed that these genes do not localize to a specific area within the oocyte, demonstrating a key distinction between axolotl germ cell specification and that of the model frog system, *Xenopus*. Spatial and temporal expression data was consistent with our working hypothesis that salamanders show marked similarity with mammals in early embryonic development.

It has been proposed (see Michael, 1984) that required maternal factors might be widely distributed, and germ cell formation takes place when cells containing these factors occupy appropriate sites in the embryo. Our data do not refute this hypothesis. In this scenario, GP components could act according to the conventional view as agents that "protect" germ cells from the action of patterning signals that would specify them to a somatic fate. Alternatively the GP components could act positively to mediate the germ cell fate in response to signals form adjoining tissues. However, given the low abundance of *Axdazl* and *Axvh* transcripts, and potentially other GP components as well, it is also possible that GP or nuage has little function in the embryo, but is a remnant of a period of relatively high expression and important function in small oocytes during oogenesis. Further work testing the effect of depletion of maternal *Axdazl* and *Axvh*, and other possible GP components that we identify in axolotls, will be required to resolve these alternatives.

Another key gene involved in regulative germ cell specification is *Axoct-4,* an ortholog of mouse *Oct-4*, which was isolated in our laboratory in a related project carried out

by Dr. T. Masi. Interestingly, *Oct-4* is expressed in the mouse inner cell mass (ICM), epiblast, and in female germ cells throughout most of the life cycle (Nichols *et al.,* 1998; Pesce and Scholer, 2001). It is also required in embryonic stem cells to maintain the pluripotent state (Niwa *et al*., 2000). Characterization of *Axoct-4* in axolotl embryos revealed that it is not expressed in migrating PGCs even when germ cells are marked by the expression of *Axvh* and *Axdazl*. However, during development *Axoct-4* expression is enriched in the presumptive dorsal mesoderm, with the highest level of expression near the dorsal lip in the axolotl gastrula (Masi *et al*., 2001; Bachvarova *et al*., 2003) the same region that will give rise to the germ cells.

Comparative studies of germ cell determination have show that the mechanisms for specifying the germ lineage are more similar in urodeles and mammals than they are in urodeles and anurans. We proposed that the evolution of the genes that are associated with germ cell formation reflect a mechanistic phylogeny rather than the organismal phylogeny, and that this relationship is due to the retention of the primitive condition found in both taxa (Johnson *et al.*, 2003). Data present in Chapters 2 and 3 confirm this hypothesis by demonstrating that *Axdazl* and *Axvh* transcripts show marked spatial and temporal similarities with their mammalian orthologs. Moreover, we were able to demonstrate that it is possible to induce ectopic PGCs using controlled conditions that include cloned growth factors (Chap. 4). Finally we provide results suggesting the gene products of vertebrate *vasa* homologs show a peculiar relationship that follows the evolution of a developmental process, instead of a taxonomic relationship.

In animals with regulative germ cell specification, the germ line arises anew at each generation from a pool of totipotent precursors also capable of commitment to somatic development. In conclusion, we have developed new molecular reagents for axolotl embryo research and showed how this system can be used to examine fundamental aspects of conserved developmental processes. This work opens new avenues of research into questions of how embryonic pattering signals maintain a population of totipotent cells, with possible implications for understanding fundamental questions of genetic continuity from one generation to the next.

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