Chapter 1

Background

Chordates are a branch of deuterostome that are characterized by a dorsal nervous system, pharyngeal gill slits, and defined by the presence of a notochord. Tunicates are one of the three subphyla of chordates and are grouped because of their outer covering known as a tunic. During development tunicates form a tailed larvae that closely resembles the vertebrate body plan [10] and this tadpole larvae is typical of ˜3000 tunicates *cite*. Out of these 3000 species 16 are known to have independently lost their larval tail, with the majority of them being *Molgula* [1, 32]. During this time, known as the free-swimming stage, the elongation and mobility of the tail is depended upon the proper formation of the notochord and muscle cells [28]. As a tissue the notochord is closest related to cartilage and serves as the axial skeleton of the embryo in addition to a source patterning signaling [12]. In ascidians and in lower vertebrae the improper formation of the notochord leads to severely shortened larva that cannot swim or feed properly [5, 13, 29]. We present a comparative study of the tailed *M. oculata* and the tail-less *M. occulta* through gene expression in order to understand the underlying factors behind tail development and tail loss.

Ascidians are a simpler system to study developmental processes, their development is well studied, they have invariant early cell lineages, a small number of cells [17] and there has been no documentation of ascidians developing without an invariant cell lineage [18]. In *Ciona intestinalis* there are 2,600 cells, 36 of them being muscle, 40 of them being notochord and many of these cells have be traced starting at fertilization. Tunicates have a

small number of cells compared to vertebrates, they also have rapid embryogenesis, compact genomes, few larval tissue types, simplified larval body plans and shallow gene networks [3, 10, 4]. For all of these reasons tunicates make great models for both tail development and loss, in addition to several Molgulids independently losing their tail and two of the Molgulids, a tailed and tailless species having the ability to hybridize [11].

Tail development has been previously studied in ascidians and other chordates, with no one factor being the cause of a improperly form tail or lack of tail.

Chapter 2

Literature Review

2.1 Chordate tail development

Ascidians are known for the bilateral and invariant cell cleavage. Their development well described up to the gastrulation stage[25, 26, 24]. Like vertebrate chordates such as xenopus ascidians depend on maternally localized determinates to regulate cell moments and division, however the location and identity of these determinates are different although the development of the early body plans are similar [18]. In ascidians the first cell division is coordinated by β-catenin which activates the vegetal gene and restrict GATA4/5 [] and determines the axis of division []. The notochord is one of the most distinguishing characteristics of chordates. Solitary ascidians notochords typically come from two cell lineages, the primary notochord coming from the ?A? blasomere and the secondary notochord comes from the ?B? blastomere [25]. At this stage the blastomeres are labeled in Conklin [2] convention; ?a? and ?A? for the anterior animal and vegetal blastomeres, respectively and ?b? and ?B? for the posterior animal and vegetal blastomeres, respectively. Although the notochords cells have been traced back to this point, notochord inductions does not occur until the 32-cell stage, where the notochord/nerve chord precursors are activated by fiber growth factor (FGF) and without FGF activation the cells lose competency and the notochord can no longer from [23, 22]. By the 64-cell stage there are 10 notochord cell precursors, the 8 primary precursor notochord cells are identifiable and no longer multipotent, while the 2

secondary notochord cells are not restricted until the 110-cell stage [26, 39, 16]. Two addition stages of cell division occur, one at gastrulation and one at neurulation, ending with 40 notochord cells, which is typical of most solitary ascidian tadpole larvae [2]. At the onsite of neurulation the notochord begins to form, this process includes the closing of the neural tube and posterior movement of the notochord and muscle cells, followed by the polarization and intercalate mediolaterally to the midline through a process known as convergence and extension where the cells [30]. At this point the larval tail is constructed of a notochord flanked by 3 rows of muscles on each side, and both notochord and muscle cell derive from the same blastomeres [26]. The arrangement of the notochord cells is a stochastic process, the anterior 32 cells—primary notochord cells—are always formed by the A7.3 and A7.7 blastomere and the posterior most 8—secondary—notochord cells are always formed by the B8.6 blastomere, but the ordering of the 32 most anterior is not determinate, cells from both the A7.3 and A7.7 intercalate in a random order [25, 26, 21, 30, 14]. This process, along with muscle cell are the causes the larval tail to form [21, 9, 30]. Although a tailed larvae is typical of most ascidians, several species with in the Stolidobranchia order have individually undergone tail-loss, many of which fall in the Molgulidae [1, 12, 8, 19]. The tail-less?anural?species develop in a similar manner and are indistinguishable from the tailed?urodele?counterparts up to late gastrulation [1, 32, 9]. Anural ascidians lack several urodele features including a converged and extended notochord, muscle cells and the otilith sensory organ. The absence of differentiated muscles cells and intercalated notochord are the cause for the lack of tail in these species [21, 32]. *M. tectiformis* notochord cells do not divide again after the 10 precursor cells are formed and *M. occulta* stops dividing after 20 cells [12]. The same occurs in *M. bleizi*, however after the 20 notochord cells are formed, the embryo attempts to make a tail but never does so [35]. It has also been shown that chordate embryos without fully

developed notochord and/or muscle cells do not fully elongate or fail completely to develop a tail [12, 36, 29]. Seeing that most ascidians have tailed larvae and that the tail can be restored through the use of interspecies hybrids, the lack of tail has been shown to be a loss of function. M. oculata (urodel) and M. occulta (anural) both of the Roscovita clade have been shown to produce hybrids in lab conditions. Of the known *Molgula* species *M. occulta* and *M. oculata* are the only two that can hybridize. Although *M. occulta* and *M. oculata* have been found to dwell in the same habitat, hybrids have not been found in nature and have only been produced in lab conditions, and no other crosses are known to produce hybrids. Fertilizing *M. oculata* eggs with *M. occulta* sperm in most cases produce embryos with fully formed tails. The reciprocal hybrid produces an embryo with 20 notochord cells like *M. occulta*, however the notochord cells converge and extent like *M. oculata* [32]. The ascidian tail has been shown to form in the presence of notochord and the absence of muscles cells [21] and the hybrid tail is not flaked by muscles as that of tail species [35], however in hybrids embryos that express the p58 which is associated with cytoskeleton develop urodele features. Hybrid embryos that develop urodele features are batch specific and features are only restored in embryos that express p58 [31, 9]. It was also shown that in hybrid embryos in which urodele features were restored, the number of cells that express acetylcholinesterase (AChE) in a vestigial muscle cell lineage increased [11].

As stated above the notochord is specific at the 64 cell stage. At this point *brachyury* is expressed first weakly in the at the 64-cell stage in the notochord/nerve chord precursors [38] and unlike other chordates *bra* is expressed exclusively in the notochord cells.

The Planar Cell Polarity (PCP) pathway is involved in cell movement during this process and mutations in *prickle*—a known PCP gene—have shown to cause a shortened ascidian tail

affecting both the mediolateral intercalation and the elongation of the ascidan tail[13]. The *pk* mutant *aimless* produces a truncated tail, however the polarity of the nuclei are present, showing that prickle does not establish polarity with in the cell but polarity between cells [13]. However, the PCP"However, even in the absence of the PCP pathway considerable convergence and elongation of the notochord was observed in Ciona, driven by a presumed boundary effect" [37].

The tailless *Molgula* develops in a similar manner and is indistinguishable up to gastrulation. Notochord cells are always present in the tailless *Molgula*, however, the cells never seem to differentiate and go through the convergence and extension process.

2.2 Known genes

Ascidians and vertebrates such as xenopus both depend on maternally localized determinates however the location and identity of these determinates are drastically different although the development of the early development of their body plans are similar [18]. Maternal factor start throughout the ascidian embryo and migrates to the vegetal pole just after fertilization, whereas in the xenopus embryo the vegetal pole is the starting position for maternal factors. For ascidians that initial maternal factor is *β-catenin* which activates the vegetal gene and restrict GATA4/5/6 expression.

there are 3 major pathways in chordates: FGF, BMP and Nodal.

Brachyury is a known notochord inducer and in ascidians only expressed in the notochord cells [7, 36]. Without *bra* the ascidian tail does not form. Although *bra* is necessary, its presence does not guarantee a tail. *M. occulta* and *M. tectiformis*, two tailless *Molgula*, both express bra. In both cases bra expression stop earlier than that of *M. oculata*, but produce

different results. *Bra* is expressed in the 10 precursor notochord cells in *M. occulta*, another round of cell division occurs which does not in *M. tectiformis*. In these two species of *Molgula* muscle actin became pseudo genes and however the mutation in the muscle actin genes are not the same [35, 12]. *Manx* is another gene identified to be important for tell development in *Molgula*, however, not in all ascidians. *Manx* is lowly expressed in *M. occulta*, and has been shown to restore the hybrid tail, but there is no homolog for *manx* in *C. intestinalis* [33, 34].

the induction of the notochord begins at the 32 cell stage by fibroblast growth factor (FGF) in the A6.2 and A6.4 notochord/nerve cord precursors[27] after the 7th cleavage. the notochord FGF transducer FGF receptor, Ras, MEK and MAPK. MAPK promotes Ets which promotes *Bra* at the 64 cell stage. If FGF is not present at the 32 cell stage competence is loss and bra is not induced. This is because is be MAPK which is down stream in the cascade is not activated and the induction of bra and repression of FoxB are not carried out [6]. And in the absence of bra notochord cell become nerve chord cells (Yasuo and Satoh 1998 Consevation of the developmental role of bra).

It was observed from isolation experiments that notochord/nerve cord precursors that loss FGF competence at the 32 cell stage assume the default nerve cord cell fate [20]

Oikopleura did not exhibit the same mechanism for tail development as Ciona, of the 50 bra target genes previously identified cite only 26 of them had orthologs in Oikopleura [15] of those genes expression ranged from notochord specific to tail including possible notochord, to tissues that were clearly not the notochord.

It was shown in H. roretzi that FoxB is represses the activation of *bra* predominately through the binding of Fox BS1 (GCACTGA*ACAAACA*TACATAG). *FoxB* is activated by *ZicN* and present in both nerve cord and notochords precursors, however is repressed by

MAPK in the notochord cell lineage at *64* cell stage [6]. MAPK is thought to be repressed by Ephin which is one of the key differences between notochord and nerve cord determination. Ephin and FoxB have redundant roles in the repression of the notochord fate, but differ in that ephin is spatial and FoxB is mediates temporal restriction of *Bra* induction.

Kourakis et al. have shown that *pk* does not establish the polarity of the within the neucli, instead *pk* directs the polarity between cell. The absence of *pk* effect the cell and its neigh, so *pk* acts in a local manner and perhaps their is a global organizer.

The notochord is derived from to cell lineages, 32 from 8 of the A-line primordial notochord blastomrers of the 110-cell embryo, the other come from the B-line notochord precusers (Satoh, , 1994;). These 10 cell divide twice giving rise to 40 notochord cell in the free-swing stage of ascidian development.

Bra is exclusively found in the ascidian notochord (Yasuo and Satoh, 1993, 1994; Corbo et al., 1997)

2.3 Hybrids

2.4 Assembling and analyzing data

One of the major advances in science in the past 20 years was the implementation of sequencing technologies. These technologies allowed us to examine problems in ways not previously possible. The first wave were mircoarrays and sanger sequencing. Mircoarrays allow us to look at a wide spectrum of genes and understand relative expression within a sample. Sanger sequencing allowed us to sequence whole genomes and