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Functions of TBX-35, CEH-51, and TCF/POP-1 in Mesoderm Specification in *Caenorhabditis elegans*

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by

Melissa Owraghi

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Dissertation Committee:
Dr. Morris F. Maduro, Chairperson
Dr. Connie Nugent
Dr. Harley Smith

The Dissertation of Melissa Owraghi is approved:

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Committee Chairperson

University of California, Riverside

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ABSTRACT OF THE DISSERTATION

Functions of TBX-35, CEH-51, and TCF/POP-1 in Mesoderm Specification
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Melissa Owraghi

Doctor of Philosophy, Graduate Program in Cell, Molecular, and Developmental Biology
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Dr. Morris F. Maduro, Chairperson

Early cells of the nematode *Caenorhabditis elegans* decide very early which types of tissues they will contribute to. The E and MS blastomeres are sister cells born at the 7-cell stage of embryonic development. E makes the entire endoderm (gut), while MS makes many mesodermal cell types, including pharynx, body muscles and the four embryonically-derived coelomocytes. The E cell is specified by two redundant genes, *end-1* and *end-3*, while MS is specified by the T-box gene *tbx-35*. However, previous studies indicate that another regulator is involved in MS specification, since *tbx-35* mutant embryos continue to make some MS-derived tissues (e.g., coelomocytes). In addition, the embryos that lack NK-2 homeobox gene, *ceh-51*, previously identified by the Maduro lab to be a direct target of *tbx-35*, also showed partial specification of MS-derived tissues. Embryos that lack both *tbx-35* and *ceh-51*, however, show a synergistic

reduction in MS-specific tissues, suggesting that TBX-35 and CEH-51 work together to specify for tissues downstream in the MS lineage.

The Wnt nuclear effector POP-1 is critical to making MS and E different from one another. The MS-to-E transformation that results from loss of *pop-1* function is well documented (Lin et al., 1995), and shows that the main requirement for POP-1 in *C. elegans* is repression of endoderm fate in MS. Recently, our laboratory has reported that E adopts an MS-like fate in *C. briggsae* embryos depleted for *Cb-pop-1* function. This and other observations prompted us to look for the requirements of POP-1 in specification of MS in *C. elegans*. We examined *pop-1; end-1,3* mutant embryos and found that although such embryos lack endoderm (as expected), they generate additional tissues that are normally made by MS, specifically pharynx muscle, body wall muscle, and coelomocytes. Using a laser microbeam to isolate MS or E, we have found that both cells make these tissues in *pop-1*; *end-1,3* mutants. However, E isolations in the triple mutant embryos also express the hypodermal marker *nhr-25*::YFP, suggesting that E generates some C-like tissues in the absence of *end-1,3*. Collectively, these results demonstrate that E is only partially restored to an MS-like fate. The conclusion is that POP-1 is dispensable for some aspects of MS specification in *C. elegans end-1,3* mutants, and that there may be a role for POP-1 in blocking MS fate in E.

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Chapter 1: Introduction

The dissertation examines the general question of how cells in a developing embryo acquire characteristics that allow their descendants to contribute to a restricted set of tissues. In this introduction, I will discuss how the fate of cells become specified in the early embryo of various model organisms, including *Caenorhabditis elegans*; the significance of gene regulatory networks; the advantages of using *C. elegans* as a model organism; the contributions of maternal factors to endomesoderm specification in *C. elegans*; and a general overview of the Wnt and Notch/Delta signaling pathways and their contribution to *C. elegans* embryonic development.

Cell fate specification

During embryonic development, most animals establish a pattern throughout the body plan as a result of cell-cell interactions, as well as maternal inheritance and other lineage-autonomous mechanisms which will be described later. The process of induction takes place during the cell-cell interactions and is a phenomenon that is essential for cells to acquire their specified fate (Sethi et al., 2009). The fate of a cell is defined as the 'outcome' of the cell; the type of tissue the descendants of the cell will give rise to in the process of normal development. When studying the fate of a cell during embryonic development, a question that usually arises is, what structures will the descendants of that cell give rise to in the developing embryo? To answer this, fate maps are constructed in which dyes (i.e., Nile blue) are used to label and follow the descendants of a particular cell or a cell of a transparent embryo may be followed without the use of a dye. This

technique will allow for determining what structures/tissues the cells will become a part of. In some cases, cells have the 'potential' to adopt different fates or give rise to a complete organism if isolated from the embryo (Fig. 1B). However, in animals this is usually only possible very early in development, for example during the 2- and 4–cell stage. As development continues, the developmental potential of individual cells decrease as their fate becomes more restricted.

The differentiation process follows determination, in which cells that have been specified to adopt a particular fate give rise to a specific subset of tissues, for example muscle, gut, skin, and nerve cells (Powell, 2004). But one may ask, how does a group of cells in a particular region become specified? Do the cells rely on neighboring cells to carry out their fate or are the cells able to 'specify' a particular fate on their own (autonomously)? The fate of cells can be specified early in development as a result of induction through cell-cell interactions or they can be autonomously specified. In other words, some cells may already have all the information needed to execute a particular fate from the beginning and do not require any action or information from neighboring cells.

A well-characterized species that undergoes such a process during early embryonic development is the ascidians (or tunicates/sea squirts), which are a member of the subphylum, urochordates. They are a classic example of what is called "mosaic development" (Fig. 1A) (Clonkin, 1905). If a particular cell is removed or ablated a partial embryo will result and there is no compensation for the lost cell. Simply, the fates

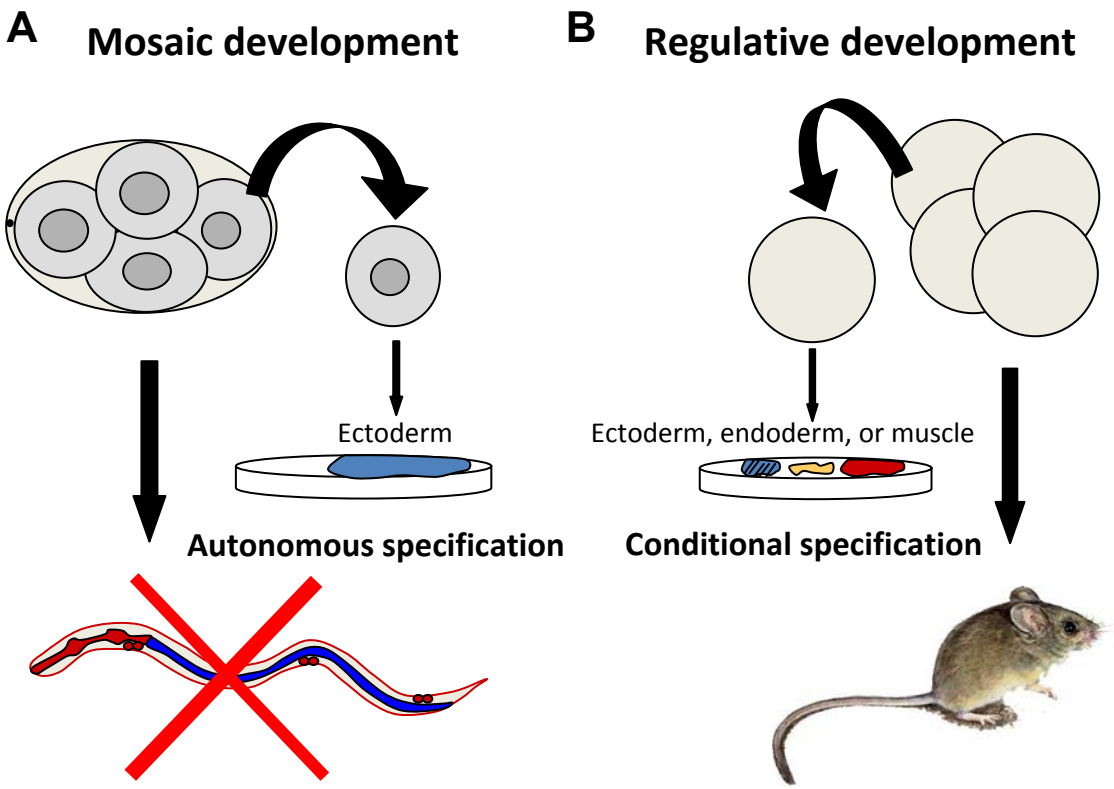


Fig. 1. Two types of developmental pathways involving different cell specification mechanisms. (A) In *C. elegans* , when a cell is removed and isolated from an early embryo (i.e., at the four-cell stage), the cell will give rise to the type of tissue it would normally give rise to in an intact embryo, thus the cell already contains its own factors and regulators to specify for a particular fate. This phenomenon is known as autonomous specification. As a result, mosaic development is a they type of developmental mechanism that occurs in which the early, partial embryo fails to develop into a complete adult worm, since the remaining cells fail to compensate for the loss of the cell. (B) In regulative development, however, if a cell is removed or killed in an early embryo the remaining cells are able to compensate for its loss and will make the tissue that particular cell was supposed to make. Typically this developmental mechanism occurs in vertebrates and the cell or cells that are isolated from the early embryo have the potential to give rise to many different tissue types, depending on the surrounding conditions, hence this phenomenon is called conditional specification.

of all the cells in the early embryo are fixed at or before fertilization. Usually this coincides with autonomous specification in which isolated blastomeres develop according to their normal fate in the intact embryo. Cells that are autonomously specified do not require cell-cell signaling in order to acquire a specified fate. Rather, these cells have intrinsic properties, for example cytoplasmic determinants, that give rise to a specific fate during cleavage. A way to study autonomous specification is to disassociate embryonic cells each time they divide, continuously, from the beginning of development until fate restriction occurs (Nishida, 1997). For example, Nishida (Nishida, 1992) continuously dissociated embryonic cells from the first cleavage to the 110-cell stage of the ascidian embryo and allowed the cells to develop into partial embryos. After the 110-cell stage, the cells were not disassociated and development continued in partial embryos. It was found that by the 110-cell stage the fates of the blastomeres are limited to give rise to a single cell type in intact embryos (i.e., mesoderm, endoderm, or ectoderm) (Nishida, 1997). The results found by Nishida demonstrated that fate specification and the initial events of differentiation of epidermis, muscle, and endoderm cells occur autonomously, independent of cell-cell interaction. Experiments by Nishida also revealed the presence and localization of maternal determinants in the ascidian egg that give rise to mesoderm, endoderm, and epidermis suggesting that maternal factors are responsible for the autonomous specification of these cell-types (Nishida, 1997). However, it was also found that some cell types, such as notochord cells, failed to differentiate when embryonic cells were dissociated, suggesting that cell interactions are required for notochord development (Nishida, 1997). Therefore, the studies demonstrate that

although an embryo may undergo mosaic development, the process may include a combination of autonomous or non-autonomous (i.e., cells depend on cell-cell interactions) cell fate specification.

In addition to ascidians the early embryonic development of leeches, which are a member of the large annelida phylum, is mosaic (Bissen, 2005). Similarly found in ascidian embryonic development, however, the later stages of leech embryonic development require fates to be specified through cell-cell interactions demonstrated by a series of experiments involving ablations by injection of toxic enzymes (Blair, 1983) or injection of photosensitizing fluoresceinated lineage markers (Shankland, 1984). Collectively, early embryonic development may be mosaic in which the cells are autonomously specified, but at some point an embryo may require cell-cell signaling in order to specify later features of development.

For about 100 years, nematodes, particularly *Caenorhabditis elegans*, have been found to be a well-known example of mosaic development (Herman, 1995; Yochem and Herman, 2003; Yochem et al., 2000). Most nematodes are characteristic of having an invariant cell lineage and have a small and fixed number of cells during embryogenesis. Within the past two decades, however, studies have revealed that the blastomeres of early embryos are induced or inhibited by adjacent cells to adopt a specified fate (Priess and Thomson, 1987; Schnabel, 1997), suggesting that they undergo a combination of autonomous and non-autonomous cell fate specification similar to other metazoans. In order to better understand the mechanisms of cell fate specification, other related nematodes have been studied; especially to determine whether the mechanisms remain

similar within the related species. Interestingly, the mechanism of cell fate specification in the closely related soil nematode *Acrobeloides nanus* has been found to be "regulative" unlike the mosaic type of development in *C. elegans* (Wiegner and Schierenberg, 1999). In regulative development, cells in the embryo are not fixed to specify a particular fate before fertilization, rather if a cell is removed early it may be replaced and the embryo will still be able to develop normally and result in a complete organism (Fig. 1B). For example, loss of a selective early blastomere in *A. nanus* resulted in neighboring cells to compensate for the loss of the ablated cell, since the neighboring cells are known to have the potential to give rise to the specific subset of tissues that were missing (i.e., gut) (Wiegner and Schierenberg, 1999). This phenomenon did not occur in less than 50% of the embryos in which the particular cell was ablated, possibly due to the fact that the cell was still able to send inhibitory signals to the neighboring cells (which occurs during normal development) regardless of ablation by a laser microbeam (Wiegner and Schierenberg, 1999). However, the remaining portions of embryos were shown to have developed gut, even though the cell that normally makes gut was ablated and, in some, the embryos were able to develop to larvae (Wiegner and Schierenberg, 1999). These findings are very different from similar studies that were done in *C. elegans*, for example, in which ablation of a cell would not result in other cells to compensate for its loss (Sulston et al., 1983) (Fig. 1A). Vertebrate development, on the other hand, is highly regulative and is usually found to have a variant cleavage pattern.

In contrast to invertebrates, in which blastomeres of an embryo are specified as early as 2- to 4-cell stages, dependent on the spatial organization of the cell lineage, cells

of vertebrate embryos are specified as a result of diffusible morphogens and cell migrations (Davidson, 1991). This mode of commitment is classified as conditional specification, in which the fate of a cell depends upon the 'conditions' in which the cell finds itself (Gilbert, 2000). If a blastomere is removed from an embryo, it can possibly give rise to several different cell-types if grown separately in culture. In addition, if a cell is ablated or removed from an early embryo that uses conditional specification, the remaining embryonic cells have the capacity to change their fates so that the roles of the missing cells can be compensated (Gilbert, 2000). This type of specification results in embryos that undergo what is known as regulative development (Fig. 1B), as explained above. Although some early specification events do occur in the developing vertebrate embryo, the fates of the cells are largely specified once after migration and reorganization of cells form into multilayered structures as a result of gastrulation (Davidson, 1991). This characteristic may be one explanation as to why vertebrate embryonic development is highly regulative.

During vertebrate embryogenesis one of the first specification events that occur is the formation of the three germ layers. Formation and specification of mesoderm has been extensively studied; however how endoderm is initially specified has long been a question in vertebrate development. Initial formation of endoderm has been studied within the past decade, particularly in *Xenopus* (Horb, 2000). One of the first factors that establish the endoderm fate is the presence of maternal determinants, specifically the T-box gene *VegT* (Horb and Thomsen, 1997; Zhang and King, 1996). Several different studies from different vertebrate model systems (i.e., chick, frog, and mouse) demonstrate

that later in development signals from the mesoderm then specify the pattern of endoderm and lastly, morphogenesis occurs as the mesoderm and endoderm attach to one another (Horb, 2000). Collectively, vertebrate cell fate specification occurs later during embryogenesis and is highly regulated through cell-cell interactions and the presence of local determinants. On the other hand, invertebrate cell fate specification is commonly found to occur as early as the 4-cell stage and usually specified autonomously or by intrinsic factors present before fertilization (i.e., maternal factors) (Davidson, 1991).

Gene regulatory networks

Properties

The mechanism of embryonic induction is critical to understand as it is helpful in clinical advancements, especially in regenerative medicine, in which pluripotent stem cells can be induced to differentiate and acquire a desired cell fate (2009; Tuch, 2006; Ulloa-Montoya et al., 2005). The genome sequence of some eukaryotic species has been made available to help researchers determine the identity and expression of transcription factors. However, it is also crucial to understand the mechanisms behind gene regulation and the interaction between the gene products which eventually determines the outcome of the induction process (Sethi et al., 2009).

The underlying mechanism of the development of animal body plans is the localized expression of encoding sequence-specific transcription factors at specific times and places (Levine and Davidson, 2005). The units of control are clusters of DNA sequence elements that serve as target sites for transcription factors, which usually

correspond to enhancers, in addition to silencers and insulators, collectively referred to as "*cis*-regulatory modules" (Levine and Davidson, 2005). In general, a particular *cis*-regulatory module produces a specific pattern of gene expression in space or time, and multiple modules can produce complex patterns of gene expression (Howard and Davidson, 2004). The *cis*-regulatory modules are usually several hundred base pairs long and are located within a few kilobases of the exons or introns of the gene they control, although some can function at distances as big as 100 kb (Howard and Davidson, 2004). Multiple transcription factors regulate each module and each transcription factor binds to multiple modules. For instance, many binding sites may be present for ubiquitous DNA binding proteins, whether the protein is involved in DNA looping or required for interaction with the basal transcription apparatus (Howard and Davidson, 2004). More specifically, a module, on average, will have several binding sites for four to eight different transcription factors (Arnone and Davidson, 1997); the more binding sites present for a given factor within a module, the greater the sensitivity will exist for that given regulator (Howard and Davidson, 2004). Therefore developmental patterns of gene expression can be represented as an interlocking network referred to as the gene regulatory network (GRN) (Arnone and Davidson, 1997).

GRNs are logic maps that state in detail the inputs into each *cis*-regulatory module, so that one can see how a given gene is activated at a given time and place (Levine and Davidson, 2005). The sequences of the *cis*-regulatory modules determine what inputs affect the expression of each gene, and how these inputs operate in a combinatorial manner (Davidson and Levine, 2008). The structure of the network is

constructed from numerous perturbation experiments, including injection of morpholino-substituted antisense oligonucleotides that effectively knock down mRNA translation of a specific gene (Howard et al., 2001), injection of engrailed repressor domain fusions to turn activators into repressors, or mRNA over-expression (MOE) (Kuhn et al., 2009), which are techniques primarily used in sea urchins.

Individual parts of the developmental process are generally controlled by GRN sub-circuits, and it is the design of that sub-circuit that sheds light on the basic logic of development (Davidson and Levine, 2008). An example of a sub-circuit of a GRN is the 'double-negative gate' (Fig. 2). This type of sub-network is used to establish a specific regulatory state in a specific region of the developing early embryo. Instead of having a regulatory gene acting as the primary activator, the system works by relieving a global inhibitor through localized transcriptional repression in a specific domain (Fig. 2B) (Davidson and Levine, 2008). For example, in the *Drosophila* embryo, the global transcriptional inhibitor, Tom, blocks expression of the Notch signaling ligand, Delta (De Renzis et al., 2006). However, a particular subset of cells is required to specify mesodermal tissues, therefore the repressor, Snail, prevents transcription of Tom and this results in mesoderm-specific expression of the Delta ligand (De Renzis et al., 2006). Consequently, Notch signaling is restricted to the adjacent cells where *sim* and other genes are activated and required for specification of ventral midline of the neurogenic ectoderm (De Renzis et al., 2006; Levine and Davidson, 2005).

A similar set of regulatory events also occurs during sea urchin embryonic development. In the sea urchin embryo, specification of the skeletogenic mesoderm

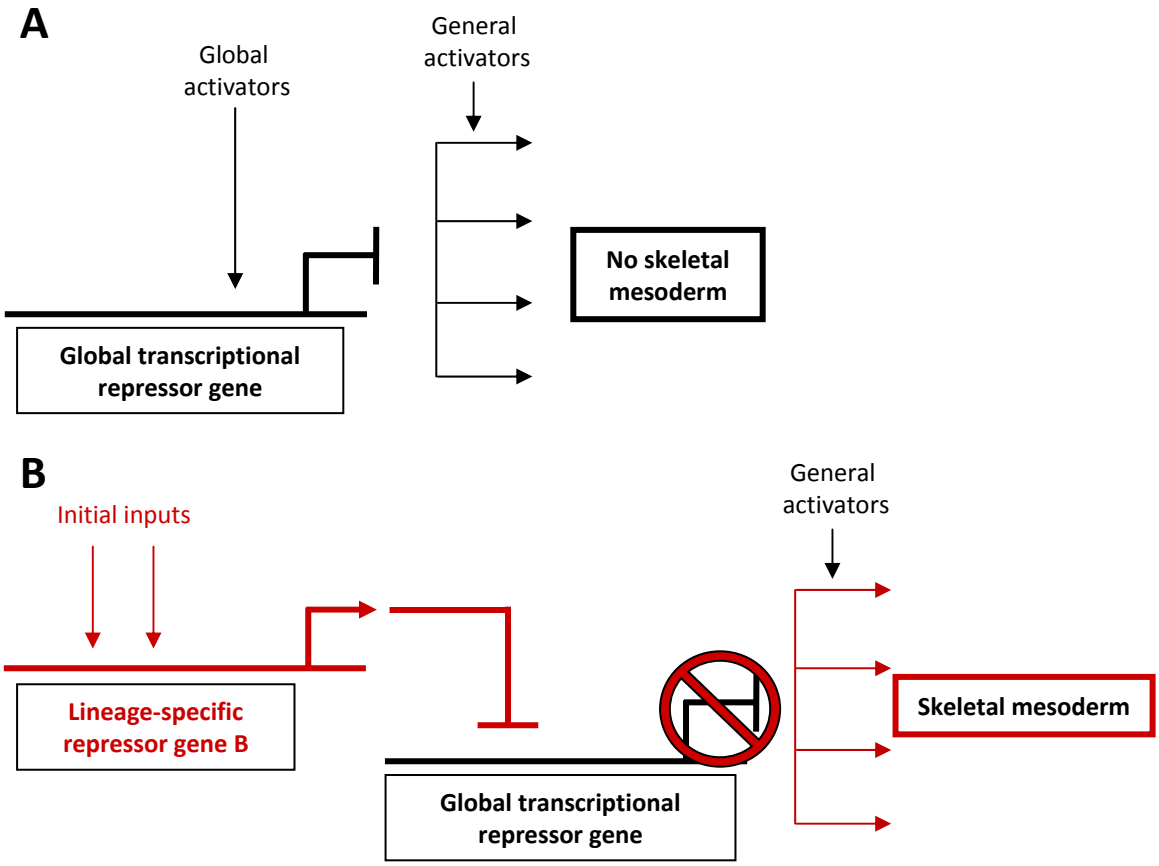


Fig. 2. A gene regulatory network sub-circuit: double-negative gate. In most GRNs, a global activator acts to regulate a specific developmental pathway (A), however in a double-negative sub-circuit a inhibitor is activated to relieve the activity of the global inhibitor in order for a particular fate to be established in a specific domain within the developing embryo (B). For instance, in *Drosophila* a global activator normally activates the global transcriptional repressor to inhibit the specification of skeletal mesoderm throughout the embryo. However, as shown in (B), a particular domain of cells in the *Drosophila* embryo requires skeletal mesoderm to be made. Therefore, in this specific lineage a repressor is activated to block the activity of the global inhibitor in order for general transcription factors to become activated and give rise to skeletal mesoderm. Figure modified, with permission, from Davidson and Levine, 2008.

involves a double negative gate sub-circuit that consists of activation of genes encoding a lineage-specific repressor, Pmar-1 (Oliveri et al., 2008; Revilla-i-Domingo et al., 2007). Pmar1, is a β-catenin-dependent determinant factor known to be transiently expressed in micromeres (located at the vegetal pole of the embryo) during the initial stages of the specification of the cell lineage (Oliveri et al., 2002). Throughout the embryo, a global transcriptional repressor functions to inhibit genes that establish the skeletogenic regulatory state. However, it is necessary for a subset of cells to give rise to skeletogenic mesoderm (Oliveri et al., 2008). Therefore, Pmar-1 is activated in those cells to prevent the expression of the global transcriptional repressor (Revilla-i-Domingo et al., 2007), and ultimately result in specification of skeletogenic mesoderm.

The sea urchin endomesoderm gene regulatory network (EM-GRN) is a well-characterized model of maternal and zygotic regulatory genes in embryonic development (Davidson et al., 2002) and is currently one of the most detailed regulatory network in existence (Gao and Davidson, 2008). The endomesoderm gene regulatory network is the genetic hardwiring of control and regulation of gene expression during development of the endoderm, mesoderm and primary mesenchyme cells (PMC) (Davidson et al., 2002). These regions mainly come from the macromeres (endoderm and mesoderm) of the embryo and micromeres (mesoderm and PMCs) (Fig. 3). As of March 2009, the sea urchin EM-GRN describes the regulation of the expression of 76 genes as well as intracellular signaling (Delta/Notch) and protein interactions (Wnt-pathway) (Davidson, 2009). In addition to characterization of the gene regulation and interactions between gene products, the changes that arise in the GRN provide insight into the evolution of.

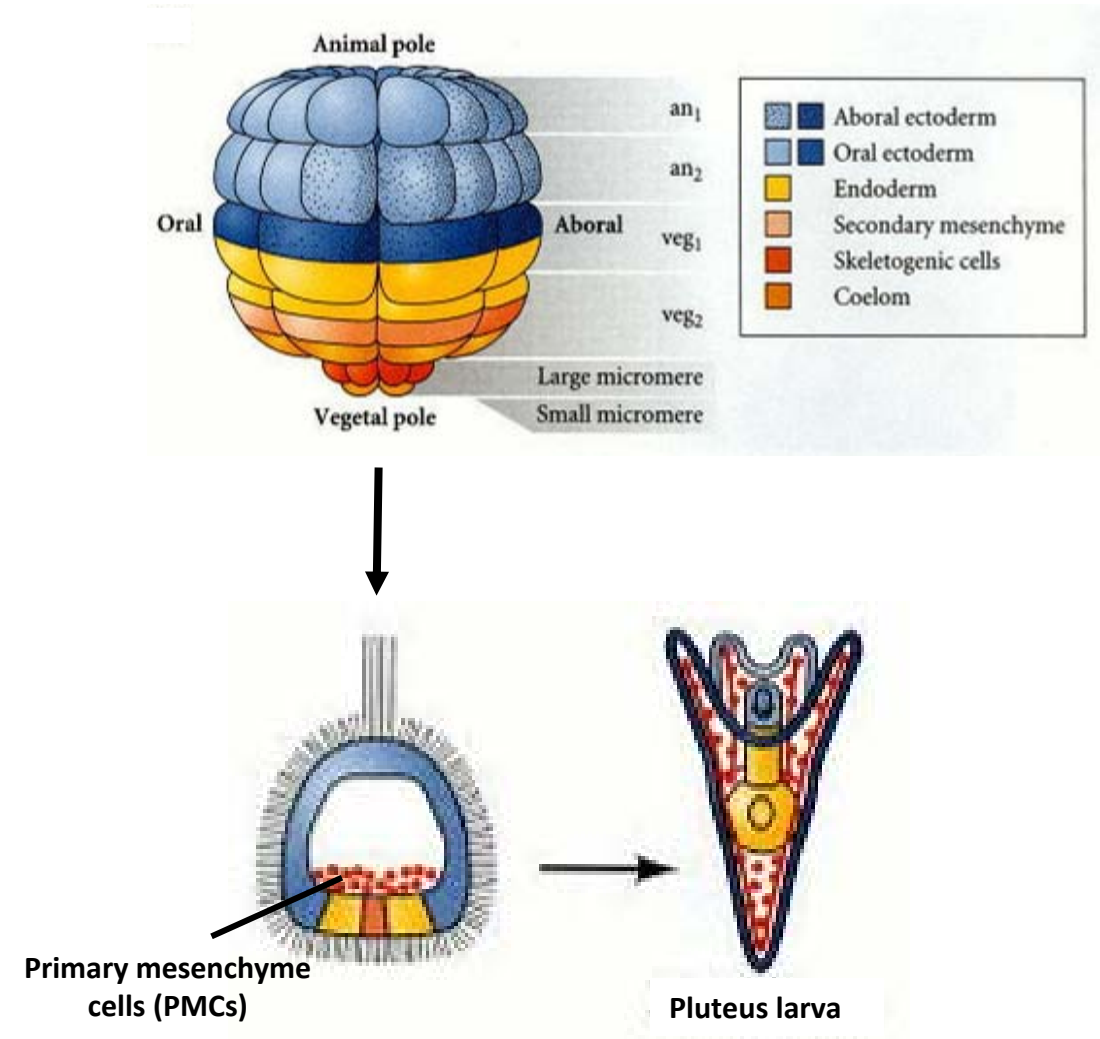


Fig. 3. Sea urchin development. The top panel is a fate map of the 60-cell sea urchin embryo in which the blastomere fates are segregated along the animal/vegetal axis. The cells continue to divide and during the developmental process the cells give rise to specified regions within the embryo, particularly the micromeres which are required for establishing the endoderm fate (orange/yellow). As development continues, the micromeres become the primary mesenchyme cells which form the larva skeleton (red). Figure modified, with permission, from Gilbert, 2000.

species-specific cell types and pattern formations that occur across distant phyla (Gao and Davidson, 2008; Hinman and Davidson, 2007)

In the sea urchin embryo, initial findings of the asymmetric localization of β-catenin across the animal/vegetal axis of the embryo (β-catenin expressed predominantly in the vegetal pole of the embryo) led to further characterization of the EM-GRN in sea urchins (Davidson et al., 2002; Logan et al., 1999). It was found that the vegetal wave of nuclear β-catenin during cell cleavages in the early embryo is required for endomesoderm specification, since the absence of β-catenin resulted in production of ciliated ectoderm with no endomesoderm tissue (Logan et al., 1999). Further findings led to characterization of other determinants involved in the endomesoderm GRN during the pre-gastrulation stage of sea urchin embryos. As previously described, Pmar-1 acts as a lineage-specific repressor and is transiently expressed in micromeres of the sea urchin embryos during the initial stages of the specification of the cell lineage (Oliveri et al., 2002). The sea urchin endomesoderm is ectopically induced through the regulatory outputs of a set of Pmar-1-responsive EM-GRN core factors, which aid in understanding how these core sets of factors will stabilize and activate downstream GRN circuits (Sethi et al., 2009).

Although the network is constantly updated, some perturbation experiments have been found to give rise to ambiguous results (Smith, 2008). The majority of regulatory connections have been described, but it is possible that more subtle features of the regulatory networks may be missing. Therefore, a group of researchers have recently performed computational analysis, specifically using a mathematical model, of the EM-

GRN in sea urchins to substantiate its interpretations and detect possible errors or ambiguities (Kuhn et al., 2009).

Redundancy

Studies of genome sequences and gene networks have shown that many functions are encoded by multiple copies of paralogous genes, which are found to be products of recent duplications, but redundancy serves other functions as well. In addition to the numerous signaling pathways that work together to regulate gene expression and tissue specification, redundant genes also contribute to robustness of a gene regulatory network. Genetic redundancy implies that two or more genes have overlapping functions and depletion of one of the genes will not result in an obvious phenotype. Recent experiments in plants and animals suggest that many genes function in complex 'information networks,' with cross-talk between regulators and/or feedback loops (Pickett and Meeks-Wagner, 1995). These interactions result in a form of redundancy that maintains the activity of a pathway and informational homeostasis, even when a member of a network is missing as a result of a mutation (Pickett and Meeks-Wagner, 1995).

Genetic redundancy has been shown to be a major feature of the genome of higher organisms (Laney and Biggin, 1996; Pickett and Meeks-Wagner, 1995; Tautz, 1992; Thomas, 1993; Wang et al., 1996). A study demonstrates that two of the four myogenic basic helix-loop-helix transcription factors, *Myf5* and myogenin (*myg*) are functionally redundant. In the absence of *Myf5*, mice fail to develop a rib cage, however when *myg* is 'knocked-in', the mice are rescued and develop a normal rib cage (Wang et al., 1996).

Since myogenin is expressed and controlled under the same regulatory elements of *Myf5*, resulting in rescue of the *Myf5* mutant phenotype, it was concluded that the proteins encoded by *myg* and *Myf5* are functionally redundant for proper rib development (Wang et al., 1996).

In *C. elegans*, redundancy is known to function in the early embryo. The endoderm specifying genes *end-1* and *end-3* encode linked GATA-transcription-factor homologues (Maduro et al., 2005a). Loss of *end-3* alone results in majority of embryos having an abnormal number of gut cells and only about 5% undergo developmental arrest, however the absence of both *end-1* and *end-3* results in complete loss of endoderm specification (Maduro et al., 2005a; Zhu et al., 1997). Another pair of GATA factors, *med-1* and *med-2*, has also been found to be functionally redundant. Although *med-1* and *med-2* are on different chromosomes, the genes are almost identical over a ~1.3 kbp region (Maduro et al., 2001), suggesting that the two genes may be functionally redundant. It has been reported that the gene expression of *med-1* and *med-2* occur at, relatively, the same time at the 4-cell stage (Maduro et al., 2007). However, the absence of *med-1* and *med-2* leads to embryonic arrest with loss of posterior pharynx, other MS (*m*e*s*odermal blast)-derived tissues (i.e., MS-specific muscle, coelomocytes), and ~50% lack intestine (Broitman-Maduro et al., 2009; Maduro et al., 2007).

In *Drosophila melanogaster*, two Sox genes, *SoxN* and *Dichaete*, are expressed in the developing central nervous system (CNS) and has been a model for studying the function of Sox proteins in neurogenesis (Cremazy et al., 2000; Russell et al., 1996). *SoxN* and *Dichaete* are both expressed in the early neuroectoderm, but are uniquely

expressed in specific regions of the neuroectoderm. *Dichaete* is prominent in the ventral, region including the midline (Zhao and Skeath, 2002), whereas *SoxN* is expressed more dorsally surrounding the entire neuroectoderm (Cremazy et al., 2000). In *SoxN* and *Dichaete* single mutant embryos, a strong phenotype corresponding to their unique regions of expression is observed. However in the regions where their expression overlaps, embryos reveal a weak phenotype, specifically in the medial neuroectoderm (Overton et al., 2002). Double mutant embryos presented a strong, aberrant phenotype along the entire structure of the CNS (Overton et al., 2002). These observations, in addition to the finding that outside the DNA-binding domains of the two genes there is a sequence similarity, led to the conclusion that these genes act redundantly and is mediated by DNA binding (Overton et al., 2002). This particular example is characteristic of redundancy selected for divergent functions, in which two genes are independently important for a distinct function, but in some aspects of development the function of the genes overlap (Thomas, 1993).

One may question, how is it possible for redundant genes to have divergent functions? It has been assumed that one copy of a duplicate gene may undergo "beneficial" mutations (mutations that have a positive affect on an organism) resulting in a novel function while the other copy retains its original function and if a degenerative mutation (i.e., deletion) occurs then the gene will become non-functional (Ohno, 1970). However, a newer model suggests that it is through the fixation of degenerate mutations that duplicate genes are preserved and acquire new functions (subfunctions) over evolutionary time (Force et al., 1999; Ohno, 1970). The initial theory suggests that

properties of a gene is considered to have a single function, but this is unlikely the case since genes have been shown to encode for several functions, each of which may be controlled by different DNA regulatory elements (Arnone and Davidson, 1997; Force et al., 1999). It is through the mechanism of degenerative mutations that duplicate genes are retained and eventually obtain divergent functions. Other models propose that an individual function can be performed by several genes and that a given gene can perform several functions (Nowak et al., 1997). Also, redundant genes can remain evolutionarily stable provided that developmental error rates are higher than mutation rates in the germ line (Nowak et al., 1997). In other words, this model suggests that redundancy should be more common in developmental genes expressed in spatio-temporal patterns in the body than in genes encoding for 'housekeeping' functions required in all cells (i.e., metabolic enzymes) (Nowak et al., 1997).

Another example of redundant genes with divergent functions occurs in *C. elegans*. Two cell-surface receptors, *lin-12* and *glp-1*, are involved in cell-cell interactions in *C. elegans* development (Yochem and Greenwald, 1989). Loss of either *lin-12* or *glp-1* function results in the failure of specific cell-interactions that occur during post-embryonic development, however in the absence of both *lin-12* and *glp-1* function additional cell-interactions fail to occur during embryogenesis, which do not fail in either single mutants (Lambie and Kimble, 1991). Specifically, in the *lin-12(lf)* (*lf* = loss of function) mutant opposite aberrant cell transformations oocur in several cell fate decisions including early gonadogenesis, vulval precursor cell fate specification, and sex mesoblast specification (Greenwald et al., 1983). Loss of zygotic *glp-1* results in cells in

the germline to enter meiosis prematurely (Austin and Kimble, 1987). In addition, the absence of maternal *glp-1* activity caused failure of induction of the anterior pharynx at the 12-cell stage of embryogenesis (Priess et al., 1987). The amino acid sequence and structure of *glp-1* and *lin-12* was found to be similar and suggested that the two gene products may function redundantly (Yochem and Greenwald, 1989). In *glp-1; lin-12* double mutant embryos, additional phenotypes were revealed which fail to arise in single mutant embryos. These include: absence of a rectum, anus and excretory cell, as well as a duplication of the excretory pore, all of which demonstrate failure of cell fate decisions during embryogenesis (Lambie and Kimble, 1991). These results suggest that *lin-12* and *glp-1* function redundantly in additional cell interactions, whereas they have distinct functions for the other cell interactions. Many other genes in the *C. elegans* genome exist as functionally redundant. It is estimated that 6,000 of the 20,000 genes in *C. elegans* can be mutated and observe a visible, lethal, or sterile phenotype (Consortium, 1998; Fraser et al., 2000; Gonczy et al., 2000; Hodgkin and Herman, 1998). The remaining 14,000 genes may encode for other transcription factors or just have non-essential functions, but some may also be 'silent' due to a gene compensation mechanism, thus contributing to the robustness of a developmental pathway.

Robustness

Biological systems are generally capable of maintaining phenotypic stability under certain conditions such as environmental changes, random intrinsic events, and genetic variation, all of which may alter and interfere with the normal behavior. This

concept is known as robustness: a property that allows a system to maintain its characteristic behavior regardless of internal or external perturbations (Stelling et al., 2004). Cells continuously become exposed to various factors, whether they are within the cell (mutations) or outside the cell (modified signaling input), which may result in a disruption in the cells normal function. However, under certain circumstances cells are able to withstand or adapt to some of these perturbations due to the complexity of signaling networks, feedback loops (control circuits), and/or genetic redundancy, for example. Examples of robustness are common throughout nature: proteins tolerate changes in amino acids, metabolic networks continue to function in the absence of intermediate steps, gene regulatory networks are unaffected by alterations between gene interactions, genetic changes during embryonic development rarely affect the viability of an adult organism, and microbes, as well as higher organisms, have the capacity to tolerate complete elimination of a number of genes (Bhattacharyya, 2009). For example, the fate decision of λ phage, which leads to the activation of the lysis or lysogenic pathway, was thought to be dependent on the binding affinity of the promoter to corresponding regulatory factors. However, further studies revealed that it is the structure of the network, which consists of both positive and negative feedback loops that is responsible for the fate decision and not the binding affinity (Little et al., 1999). These conclusions were made when point mutations within the promoter did not result in an altered phenotype, suggesting that the fate-decision is robust against such modifications (Little et al., 1999). Another biological example is in *Escherichia coli*. Studies reveal that *E. coli* are capable of chemotaxis over a wide range of chemo-attractant

concentrations due to intracellular feedback loops that ensures an adaptation mechanism and is independent of ligand concentration (Alon et al., 1999; Barkai and Leibler, 1997; Yi et al., 2000).

Signaling networks underlying animal development are well-known examples of robustness in biology (Stelling et al., 2004)—that is, the developmental direction toward the wild-type is highly resistant to both extrinsic (i.e., environmental influences) and intrinsic (i.e., mutations) perturbations (Friedman and Perrimon, 2007). As previously described, evidence of "phenotypic robustness" is when a single gene mutation (intrinsic perturbation) of a gene that is known to participate in a developmental pathway does not result in an obvious phenotype (Baugh et al., 2005b). In *C. elegans*, for example, maternal and zygotic activities of the homeodomain protein PAL-1 specify the identity and maintain the development of the C-blastomere lineage (Hunter and Kenyon, 1996), (Edgar et al., 2001). Baugh et al., have identified genes whose expression depends either directly or indirectly on PAL-1 targets and have shown that they affect specification, differentiation, and morphogenesis of C-lineage cells. However, RNAi of most PAL-1 targets does not result in a detectable phenotype. Since the regulatory network specified by PAL-1 functions alone, while controlling multiple developmental processes (Hunter and Kenyon, 1996), this suggests that the network is organized in a modular fashion and that PAL-1 targets may have overlapping or compensatory functions (Baugh et al., 2005b). Therefore, more than one mutation may be necessary to detect a phenotype due to the synergistic affect of multiple mutations in a network.

Robustness can be an unfortunate characteristic, however, specifically in cancer cells. It promotes tumor growth and survival in several ways. For example, heterogeneity among tumor cells provide high levels of redundancy, thus resulting in increased chances of growth and survival (Kitano, 2003). In addition, feedback controls at the cellular level enhance such properties in cancer cells (Kitano, 2003). Carcinomas, for example, are generally heterogeneous and intrinsically redundant populations of cells that are a result of transformations due to various mutations in individual genes, mitotic recombination, and the loss or gain of entire chromosomes (aneuploidy) (Kitano, 2003).

The heterogeneous redundant properties of cancer cells is what makes the tumors robust, because survival of these proliferating tumor cells allows the cancer to recur (Kitano, 2003). On the other hand, it may provide insight for the development of new drugs and therapies. It is of particular interest to develop drugs that target cell cycle, growth decisions, and apoptosis. Computer simulations have shown that cell cycles that are robust against certain perturbations can become fragile (vulnerable) if a specific feedback loop is removed or attenuated, thus resulting in an arrest in the cell cycle. Although this has not been experimentally demonstrated, it does suggest the possibility that robustness can be modified if particular regions of networks are selected and understood carefully (Kitano, 2003). Carlson and Doyle, have established a highly optimized tolerance (HOT) theory, which suggests that robust complex systems are robust to common perturbations, but fragile against uncommon ones (Carlson and Doyle, 2000). However, this theory has yet to be applied to biological systems such as tumors. In addition, to the application of this theory, the points of fragility and dynamics of the

regulatory networks of cancer cells require a deep and better understanding in order to use the findings to target the vulnerable components of regulatory networks with novel therapeutic drugs (Kitano, 2003).

C. elegans as a model system

Many model organisms are extensively used for research, ranging from bacteria and yeast, to fish, frogs and mice. These model organisms can provide new ideas for therapeutic medicine; shed light on cell fate specification during development; and provide a better understanding of the mechanisms of the transcription machinery. Surprisingly, 20% of human disease genes have counterparts in yeast which contain ~6,000 genes. Such human diseases result from disruption of basic cellular processes such as DNA repair, cell division or control of gene expression (Twyman, 2002d). The zebrafish is also a useful model organism in studying human diseases. It is easy to induce mutations in these organisms and large-scale screens have been done to identify the mutations that cause defects in biological processes. Zebrafish mutants have been produced and used to test candidate drugs for human diseases (Twyman, 2002a).

Although frogs and chickens are particularly not useful to study genetic mechanisms, they are helpful in providing insight in the process of early development. *Xenopus*, specifically, has been useful for analysis of events occurring early in development, such as formation of the neural plate, which give rise to the entire nervous system; while chickens have been used for elucidating the molecular basis of limb

development and cell migration in the nervous system, which has helped uncover reasons for many limb and neural defects in humans (Twyman, 2002b).

From the vast array of model organisms used today, *Drosophila* has been studied the longest: for nearly a century. *Drosophila* has a well established history and studies that have been done to date provide a wide array of knowledge in genetics and molecular biology for further research. Of the 289 known human disease genes, 60% have homologs in *Drosophila* (Rubin et al., 2000; Tickoo and Russell, 2002). Also, they are small and have a simple diet, allowing them to be maintained inexpensively in the laboratory (Twyman, 2002c). Such resources provided by the *Drosophila* model system may help provide more insight in transcription regulation, as well as therapeutic possibilities in human cancers and other disorders.

Although, *Drosophila* has been one of the major organisms representing invertebrates, the roundworm, *Caenorhabditis elegans* has also been studied extensively in the field of development biology. *C. elegans* is a very simple, transparent, multi-cellular organism that can be studied in great detail and can provide insight to molecular and genetic mechanisms in human development, as well. In 1974, research in molecular and developmental aspects of *C. elegans* was initiated by Sydney Brenner and, since then, has been used extensively as a model organism (Brenner, 1974a; Brenner, 1974b).

Since *C. elegans* is a multicellular organism, biological information that we learn from them may be directly applicable to more complex organisms, such as humans. Indeed, approximately 35% of the 20,000 predicted *C. elegans* genes (Consortium, 1998), have human homologues. The genome sequences of other related nematodes are also

available (Wylie et al., 2004), which offers researchers to compare and validate their findings with related species. Furthermore, *C. elegans* has a number of useful tools that can be used to study biology at the molecular level. For example, animals can be made transgenic using microinjection (introducing a foreign gene into the organism). In RNA interference (RNAi) knockdown experiments, dsRNA can be delivered through microinjection or worms can be fed *E. coli*, which is engineered to accumulate dsRNA (Scholars). This phenomenon was discovered in the late 90s by Andrew Fire, Craig Mello (both recipients of the Nobel Prize in 2006 for their discovery of "RNA interference–gene silencing by double-stranded RNA"), Lisa Timmons, and others (Fire et al., 1998; Timmons and Fire, 1998). In this study a particular gene of interest was used and a corresponding double-stranded RNA (dsRNA) was expressed in *E. coli* by inserting a segment of the coding region of the particular gene into a plasmid vector designed for bidirectional transcription by bacteriophage T7 RNA polymerase (Timmons and Fire, 1998). The ingestion of bacterial dsRNA by the animals activates the RNAi machinery causing degradation of the mRNA that carries the genetic information which is identical to the double-stranded RNA (Fire et al., 1998). This mechanism results in the gene of interest to be "silenced" and fails to express in the adult but more so in the progeny. Another example of "simple experimentation" is that mutations can easily be generated by chemical mutagenesis or exposure to ionizing radiation (Anderson, 1995; Jorgensen and Mango, 2002). Such mutations are sometimes designed to identify genes that participate in a particular process or allow for thorough characterization of a gene and lead to further analysis and exploration of other genes. Using a strain that contains one

mutant gene can be crossed (mated) with a previously characterized mutant strain to examine functional interactions between genes/gene products (Hope, 1999).

In the laboratory, self-fertilization of hermaphrodites or crossing with males can be manipulated to produce progeny with desired genotypes that are especially useful for genetic study. Their rapid life cycle allows for quick experimentation and mutants to be readily identified, mapped, and cloned (Hope, 1999). These animals are transparent throughout their life cycle and animals can be examined with the use of light microscopy. At the cellular level in live preparations, however, observation is more commonly done by differential interference (DIC) microscopy (Altun, 2008). A hermaphrodite can produce about 300 offspring by self-fertilization and ~1000 if it mates with males. These traits make it easy to produce numerous genotypes and phenotypes for genetic research (Donald, 1997; Wood, 1988). This simple 1 mm organism is transparent, has a small number of cells (959 somatic cells in an adult hermaphrodite) and follows a stereotyped cleavage pattern (Sulston et al., 1983) allowing for its developmental process and embryonic cell lineage to be thoroughly defined.

C. elegans development

C. elegans is a small, transparent, free-living soil nematode that lives in many parts of the world and feed primarily on bacteria which thrive on decaying vegetable matter (Fig. 4A, B). They are found across most of the temperate regions of the world and are particularly inexpensive and easy to maintain in the laboratory. To provide optimum conditions in the lab, the animals are grown in nematode growth medium

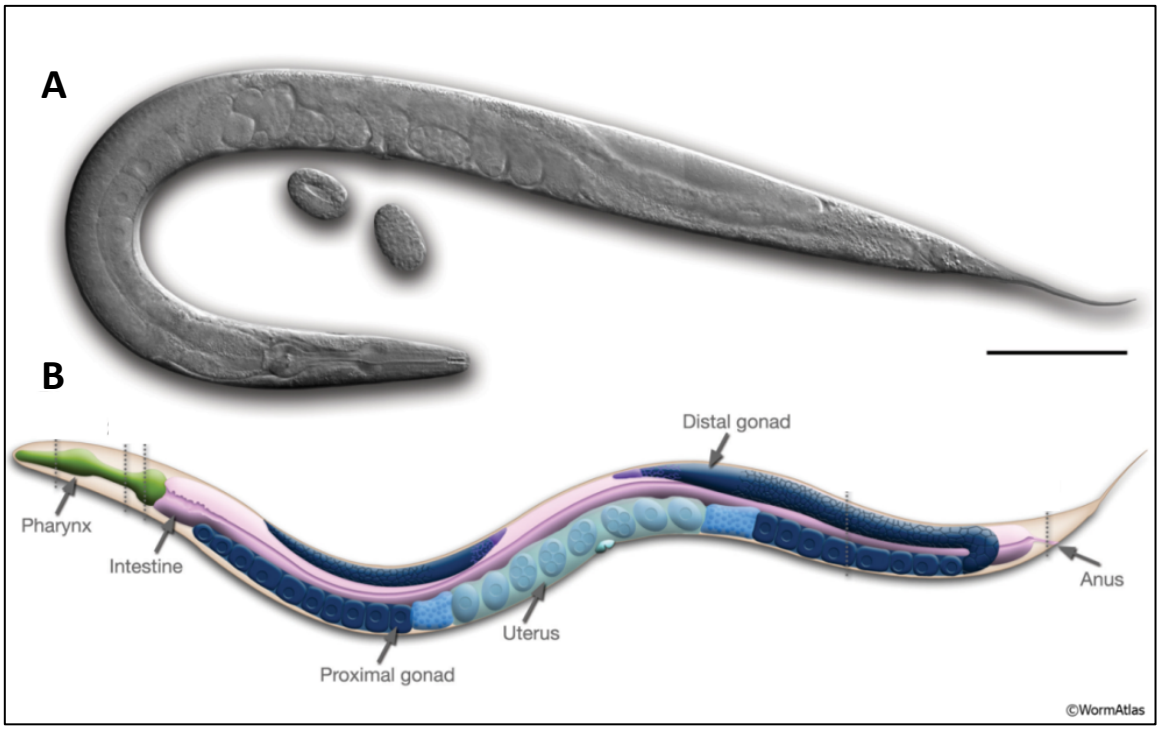
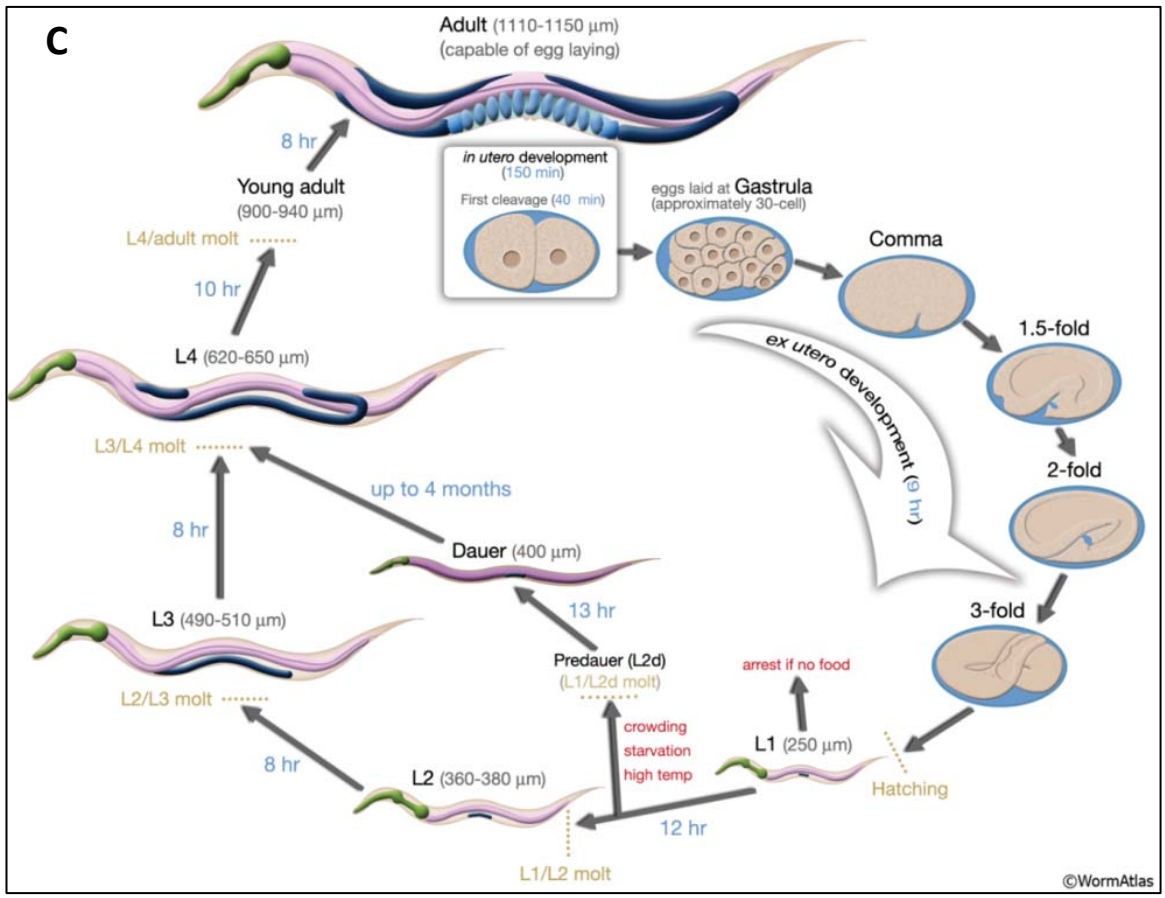


Fig. 4. Anatomy of an adult hermaphrodite. (A) Differential interference contrast (DIC) image of an adult hermaphrodite. Scale bar 0.1 mm. (B) Schematic drawing of anatomical structures. (C) **Life cycle of *C. elegans* at 22°C.** 0 min is fertilization. Numbers in blue along the arrow indicate the length of time the animal spends at each stage of development. First cleavage occurs about 40 minutes after fertilization. Eggs are laid outside the mother about 150 minutes after fertilization and during the gastrula stage. The length of the animal is marked next to the stage name in micrometers (μm) Figure composited by and legend modified from Wormatlas (http://wormatlas.org).

Fig. 4 continued



(NGM) agar plates containing a lawn of *E. coli* bacteria, which they ingest and grind in the pharynx and subsequently absorb the bacterial contents in the gut (Altun, 2008). In addition to these optimal conditions, a humid environment, ambient temperature, and atmospheric oxygen are requirements for proper growth and reproduction (Hope, 1999).

C. elegans have two sexual forms: self-fertilizing hermaphrodite (XX) and male (XO), however males arise infrequently (0.1%) by spontaneous non-disjunction in the hermaphrodite germ line and at higher frequency (up to 50%) through mating (Brenner, 1974). Under standard laboratory conditions, *C. elegans* development is very rapid. The entire life-cycle, from egg to an adult producing more eggs, takes about 3.5 days at 20ºC (Hope, 1999) [Fig. 4C; (Altun, 2008)]. However, the time range of the life-cycle varies depending on temperature: less than 3 days if animals are grown at 25ºC to 6 days at 15ºC (Byerly et al., 1976). Embryogenesis (developmental process from fertilization to hatching) takes approximately 14 hours at 20ºC, the first few hours being in the uterus of the adult hermaphrodite. Once the egg has hatched, the first larval stage is generated, which looks similar to the adult but smaller (250 μm long) (Sulston et al., 1983). Post-embryonic development continues through four larval stages (L1-L4) before the final molt to produce the adult, which is 1 mm in length. The end of each larval stage is marked with a molt, during which a new, stage-specific cuticle is synthesized and the old one is shed (Altun, 2008). In the absence of food, the animal can arrest in development (after the L2 stage), which is called the dauer stage (Fig. 4C). The dauer is a non-aging state because its duration does not affect post-dauer life span. During the dauer state, feeding is arrested and movement is reduced. The dauer state ends when the animal

experiences favorable conditions. Within 1 hour of accessing food, the animal exits the dauer stage; after 2-3 hours it starts to feed, and after about 10 hours, it molts to the L4 stage (Altun, 2008).

C. elegans cell fate specification and maternal contributions to endomesoderm specification

C. elegans cell fate specification

In the *C. elegans* embryo, the fate of the blastomeres is decided early through expression of maternal transcription factors and specific cell-cell interactions (Schnabel, 1997). Studies have shown that the invariant cleavage patterns in the embryo set up reproducible patterns of cell-cell interactions resulting in an invariant cell lineage between different embryos (Schnabel, 1997; Sulston et al., 1983). Once the egg is fertilized cell division begins and the egg is cleaved into a larger anterior (AB) and a smaller posterior (P_1) daughter cell. The fates of these two blastomeres are dramatically different and has been demonstrated by observing the lineage patterns in an intact embryo (Sulston et al., 1983). In addition, when the two cells are separated upon their initial appearance, the developing patterns are observed in isolation and it has been found that cell-cell interactions between AB and P_1 are required to give rise to certain cell-types by the AB cell (Priess and Thomson, 1987). If P_1 is removed and AB develops in isolation, the descendants of the AB cell will fail to give rise to specified cell types, including the anterior pharyngeal cells normally derived from ABa and the intestinal-rectal valve cells characteristic of ABp fate (Bowerman, 1998). This suggests that AB development

depends heavily on cell signals from the descendants of P_1, but also relies on specific factors that give rise to AB and its descendants autonomously in order for proper development to take place (Bowerman, 1998). The AB cell primarily gives rise to the hypodermis, some neurons and some contribution to the development of muscle. On the contrary, if AB is removed at the two-cell stage of the embryo, the P_1 blastomere continues to divide into the many cell types it normally makes. Therefore, P_1 has the intrinsic ability to develop, suggesting that localization of embryonic determinants to P_1 might specify its fate, with cell signals from AB descendants not having an effect (Bowerman, 1998).

After several rounds of divisions the P cell eventually gives rise to the germline and somatic daughter cells, resulting in the birth of P_4, EMS, C and D blastomeres (Sulston et al., 1983). The EMS cell divides asymmetrically and gives rise to the founder cells MS and E, which are born at the 7-cell stage of *C. elegans* development (Sulston et al., 1983) (Fig. 5). These early cleavages result in the establishment of the six founder cells: AB, MS, E, C, D, and P_4, in which the descendants of each of these founder cells have characteristic and synchronous cell cycle times (Bowerman, 1995). As shown in Figure 6, each of the founder cells contributes to specification of different tissue types in the adult nematode. In general, both cell-autonomous and non-autonomous mechanisms pattern the early embryo, with AB descendants relying more on cell signaling and P_1 descendants depending more on the asymmetric segregation of development potential during early cleavages (Bowerman, 1998).

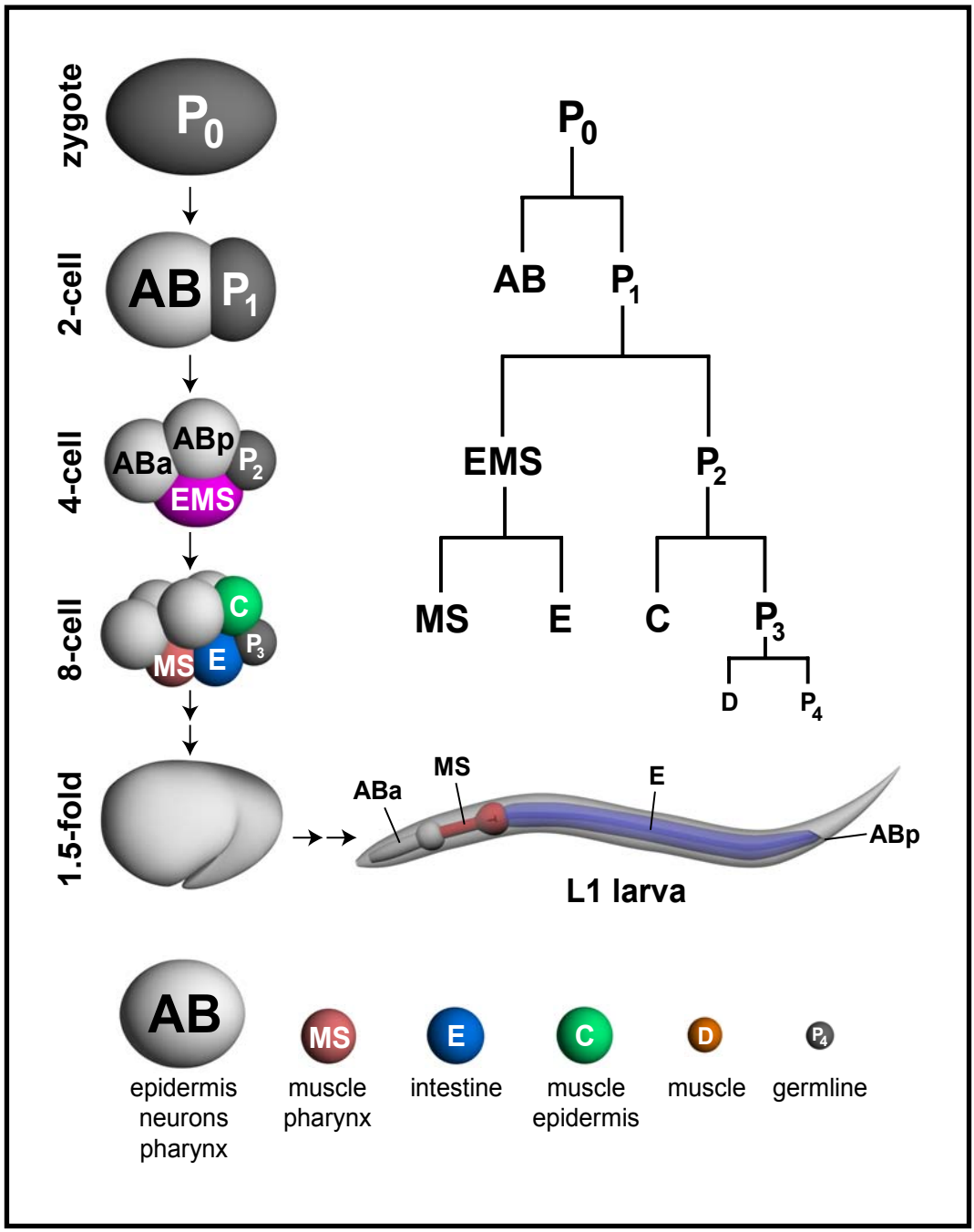


Fig. 5. The early *C. elegans* lineage showing the origin of and major tissue types produced by the six founder cells (Sulston, 1983; Maduro, 2006). Figure composited by M. Maduro (Maduro, 2006).

The mesoderm precursor cell MS and its sister cell E are distinctly different in fate as a result of the presence of maternal factors and cell-cell interactions. While E generates only a single tissue type (the intestine) derived from 20 cells, descendants of MS give rise to many mesodermal cell types, including a significant portion of the body wall muscle, the posterior portion of the pharynx, somatic gonad precursor cells (Z1 and Z4), and four blood-like coelomocytes (Sulston et al., 1983). The MS blastomere produces 80 cells, 28 of the 80 give rise to body wall muscle cells and 31 are pharynx cells which, together, comprise of the majority of the MS descendants (Sulston et al., 1983). Two pairs of coelomocytes arise from embryonic MS and another pair of coelomocytes is generated from the postembryonic M lineage, resulting in six coelomocytes in the adult hermaphrodite and five in the adult male (Altun, 2008) (Fig. 6). They are generated symmetrically in later divisions of embryogenesis when most of the other embryonic cell divisions have been completed (Sulston et al., 1983). The right ventral (ventral anterior; ccAR and ccPR) and the left ventral (ventral posterior; ccAL and ccPL) pairs are derived from MS granddaughters MSpp and MSap, respectively. Even though the mother cell for each pair is born during the time where majority of embryonic cell divisions take place, they remain arrested for about two hours before the final cell division and differentiation (Altun, 2008). Before elongation of the embryo, the two mother cells that were arrested will migrate posteriorly from the head, where they are born, to the specific locations of their daughter pairs of coelomocytes (Hedgecock et al., 1987). Migration of the coelomocyte mother cells and M blast cell during embryogenesis occurs between 250-400 minutes after the first cleavage (Altun, 2008).

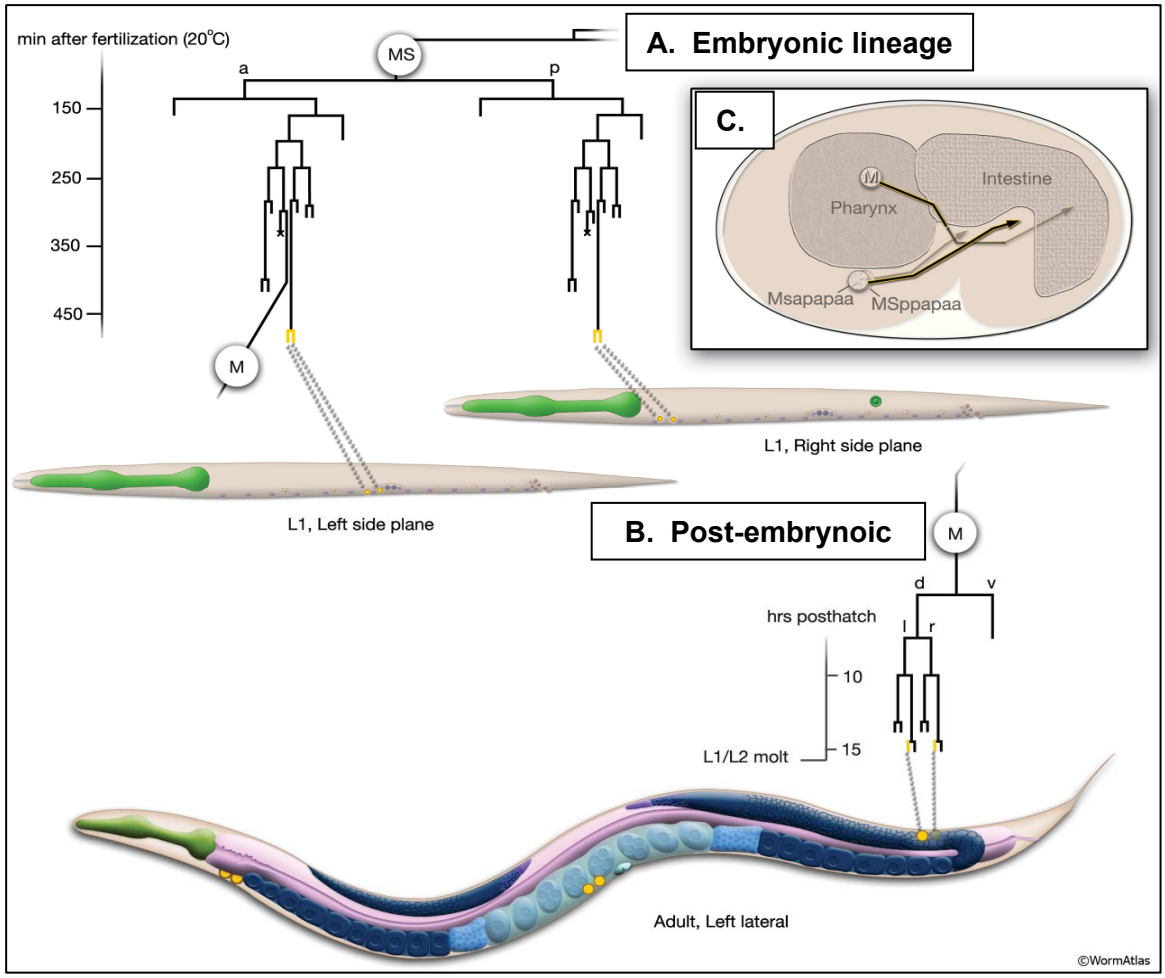


Fig. 6. Coelomocyte lineage. (A) The right ventral pairs of coelomocytes are derived from the MS granddaughters MSpp and the left ventral pairs are derived from the MS granddaughters MSap during emrbyogenesis. Although the precursors of each pair of coelomocytes are born during the beginnings of embryonic cell division, the final division is delayed until most of the other embryonic cell divisions are completed. Thus, it is one of the late MS lineage tissues that are specified later during embryonic development. (B) The dorsal pair of coelomocytes is derived from the post-embyonic M-lineage at the late-L1 stage and are differentiated into coelomocytes during early L2 stage. (C) Migration of the coelomocyte mother cells and the post-embryonic M blast cell occurs during embryogenesis between 250-400 minutes after the first cleavage. The M cell is born on the left side and migrates posterior toward the midline, eventually migrating to the right side of the animal. The right and left coelomocyte mother cells are born next to the pharynx and migrate posteriorly (Figure composited by and legend modified from Wormatlas http://wormatlas.org).

Maternal contributions to endomesoderm specification

For over 15 years many studies have elucidated the mechanisms that specify the daughters of the *C. elegans* endomesoderm precursor EMS. The result is a detailed GRN that links the earliest-acting maternal factors, through a series of regulators, to terminal differentiation genes (Fig. 7A) (Maduro, 2006; Maduro, 2009; Owraghi et al., 2009). In the event of specification of mesoderm (MS) and endoderm (E), there are two parallel processes that take place as a result of the activity of maternal gene products. One is the activation of an independent gene cascade that specifies the daughter of endomesoderm (EMS), and the other is through a signal provided by a cell-cell interaction that results in the assignment of the E fate (Maduro, 2006).

At the top of the endomesoderm GRN cascade is the maternal transcription factor, SKN-1 (Fig. 7B). A maternal gene, hence its name, is expressed in the mother but is transcribed and translated upon fertilization resulting in the gene product to be available in the egg during embryogenesis. SKN-1 is a sequence-specific DNA-binding protein with a unique C-terminal DNA-binding domain related to the basic region of bZIP transcription factors (Blackwell et al., 1994; Bowerman et al., 1993; Bowerman et al., 1992). In the absence of *skn-1*, embryos undergo developmental arrest and lack pharynx 100% of the time, while endoderm is absent 70% of the time (Bowerman et al., 1992). The fact that endoderm is still made in *skn-1* mutant embryos suggest that parallel pathways (which will be discussed in further detail below) are capable of contributing to specification of gut even in the absence of SKN-1. Antibody staining shows that SKN-1

A

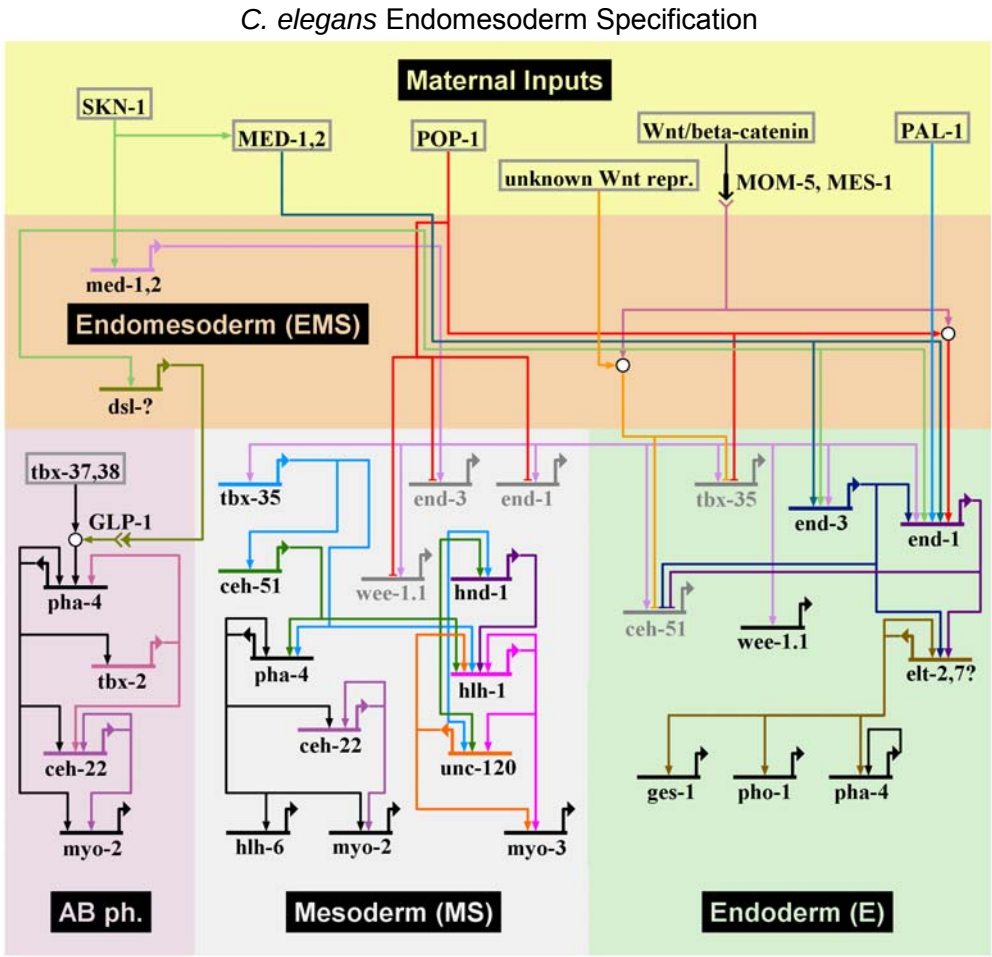
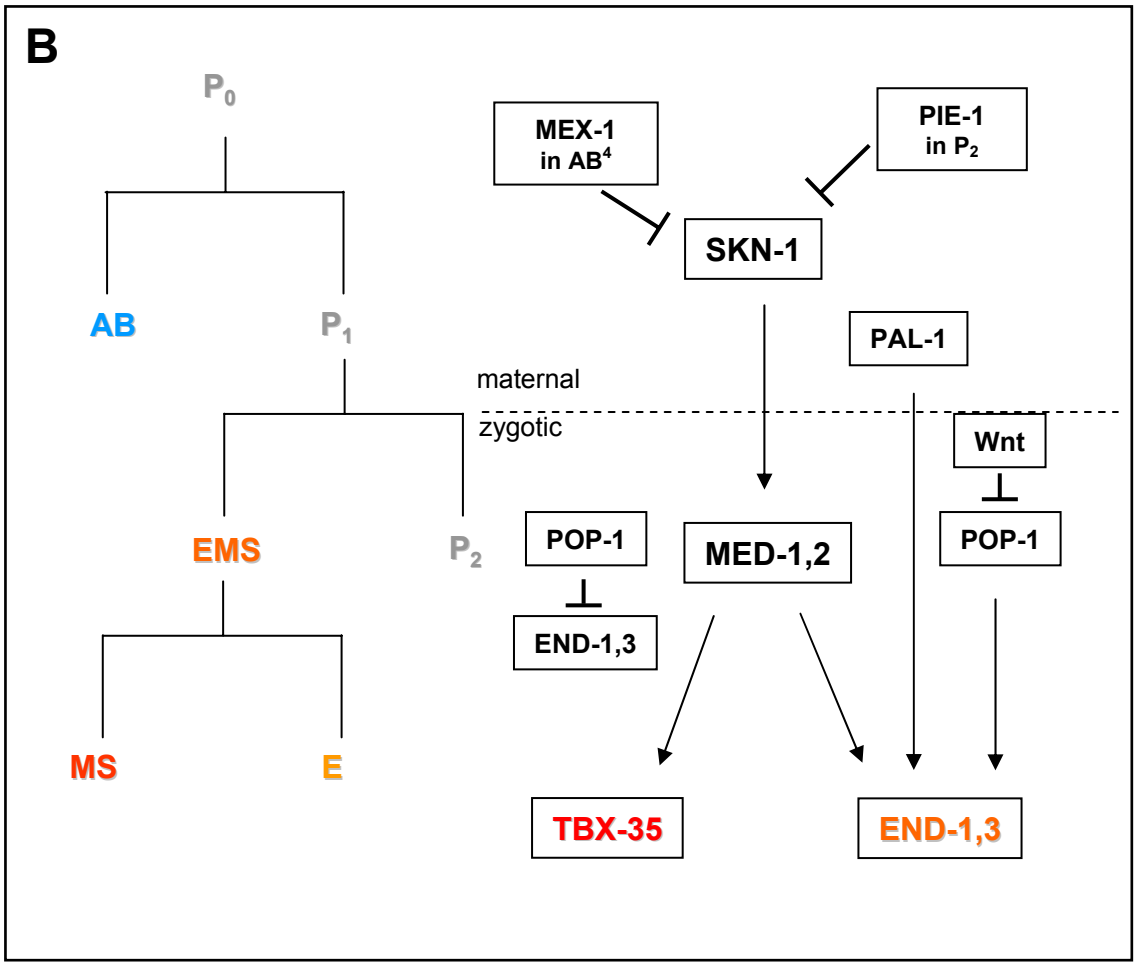


Fig. 7. (A) Updated gene regulatory network for *C. elegans* endomesoderm generated by BioTapestry (Longabaugh, et al., 2009) and modified in Adobe Photoshop by M. Maduro (Owraghi, et al., 2009). (B) Simplified diagram of *C. elegans* endomesoderm regulatory network. SKN-1 is a maternal transcription factor at the top of the regulatory cascade, in which its activity is blocked by PIE-1 in the P lineage and MEX-1 in the AB lineage, resulting in it to only function in the EMS blastomere. PAL-1 is also a maternal factor which acts to specify the C and D blastomeres. However, it has also been found for it to activate *end-1* and *end-3* in the E cell. SKN-1 binds to *med-1,2* target genes in the EMS cell resulting in its activation. MED-1,2 then activate *tbx-35* in the MS blastomere and *end-1,3* in the E blastomere. As a parallel pathway, the Wnt/β-catenin pathway is activated to specify the E fate through activation of *end-1,3* via the TCF-like factor, POP-1. As a result of these pathways, EMS divides into two asymmetric daughter cells, MS and E, giving rise to different subset of tissues. Figure A composited by M. Maduro (Owraghi et al., 2009).

Fig. 7 continued



is first detectable at very low levels in the maternal and paternal pronuclei at the end of the one-cell stage (Bowerman et al., 1993). But later, at the two-cell stage, SKN-1 accumulates at higher levels in the nucleus of P_1 than in that of AB, and SKN-1 levels peak midway through the four-cell stage in the two P_1 daughters, EMS and P_2 (Bowerman, 1998; Bowerman et al., 1993).

Although SKN-1 is present in each blastomere at the 4-cell stage, its activity is restricted to EMS. Other maternal factors, such as PIE-1 and MEX-1, inhibit SKN-1 activity in P_2 and AB cells, respectively (Bowerman et al., 1993; Schnabel et al., 1996). PIE-1 is a zinc-finger protein, present in the cytoplasm of the P_1 blastomere at the two-cell stage. This maternal factor localizes to the P_1 centrosomes and then leaves the centrosomes as P_1 finishes dividing (Bowerman, 1998). PIE-1 then disappears in the somatic daughter EMS but localizes to the nucleus in the germline precursor, P_2. PIE-1 mainly functions to repress the activation of any somatic cell fate programs in the germline precursors, thus maintaining the germline in a transcriptionally silent, inert, undifferentiated state (Seydoux et al., 1996). As previously mentioned, PIE-1 also functions to repress SKN-1 activity in P_2 (Mello et al., 1992; Mello et al., 1996). MEX-1, on the other hand, blocks SKN-1 activity in the four AB grand-daughter cells since absence of *mex-1* results in the four AB cells to adopt an MS-like fate (Mello et al., 1992). In the absence of *skn-1* and *mex-1*, however, the ectopic MS-like tissues that result in the AB grand-daughter cells of *mex-1* mutants fail to occur and ectopic hypodermal tissue results instead, suggesting that *skn-1* is required for the ectopic MS-fate in *mex-1* mutant embryos (Mello et al., 1992).

Another maternal factor first detected in the nuclei of EMS and P_2 at the four-cell stage, is the Caudal-like homeodomain protein, PAL-1. Although the mRNA is present in the oocyte, the protein expression is detected at high levels in all the P_1 descendants until after the 12-cell stage, when SKN-1 is undetectable (Bowerman, 1998). Maternal PAL-1 activity is temporally and spatially targeted to the C and D founder blastomeres by first restricting translation of maternal *pal-1* mRNA to the descendants of the posterior blastomere P_1 (EMS and P_2) and then by restricting the activity of the translated protein to the somatic descendants of P_2 (C and D) (Baugh et al., 2005a). The protein expression of *pal-1* is inhibited in the anterior blastomeres (AB cells) by the activity of the RNA-binding maternal factor MEX-3 (Draper et al., 1996). It is the combination of the activity of these maternal factors in the early embryo that establishes a controlled mechanism for SKN-1 to function strictly in the EMS blastomere to ultimately give rise to the MS and E founder cells.

Both MS and E are specified by the activity of a pair of divergent GATA-type transcription factors, MED-1 and MED-2 (Coroian et al., 2006), which are directly activated by SKN-1 (Maduro et al., 2001). RNA interference, as well as double mutant studies of both genes, have shown that *med-1,2(-)* embryos lack MS-derived tissues all of the time, while endoderm fails to be made in 15-50% of the embryos (Maduro, 2006; Maduro, 2009; Maduro et al., 2001). In addition, *med-1,2* double mutant embryos elongate only about half the size of wild-type embryos (~1.5-fold; wild-type embryos elongate ~3-fold) and arrest before hatching (Maduro et al., 2007; Maduro et al., 2001). In the MS cell, MED-1,2 activate several genes, including *tbx-35*, a T-box transcription

factor (Broitman-Maduro et al., 2006) and the endoderm specifying genes, *end-1*and *end-3* in the E cell (Maduro et al., 2005a; Maduro et al., 2001). In the MS cell, however, the activation of *end-1,3* is inhibited by a TCF/LEF homolog, POP-1 (Lin et al., 1998; Lin et al., 1995).

The *pop-1* gene encodes a protein with a previously described DNA-binding motif called an HMG (**H**igh **M**otility **G**roup) box. The HMG box in POP-1 is very similar to the HMG boxes in the vertebrate lymphoid-specific transcriptional regulators human T-cell factor 1 (TCF-1) and mouse lymphoid enhancer-binding factor 1 (LEF-1) (Lin et al., 1995). The TCF-1/LEF-1 protein function as nuclear effectors of the Wnt signaling pathway (discussed in more detail below). It is through the HMG boxes of these proteins that allow them to bind to and bend DNA, therefore, altering chromatin structure and allowing for interactions between other transcription factors (Giese et al., 1992). POP-1 is present in the nuclei of all cells of the 4-cell stage embryo, but at the 8-cell stage POP-1 levels are higher in MS than in E, suggesting that down-regulation of POP-1 in E allows for specification of endoderm (Thorpe et al., 1997). In *pop-1* mutants, MS adopts an E-like fate, suggesting that POP-1 acts primarily as a repressor in the MS cell. However, *pop-1* is known to also act as an endoderm activator in the E cell since knockdown of *pop-1* enhances the *skn-1(-)* mutant phenotype (Shetty et al., 2005). Also, in the absence of *pop-1* and *skn-1*, the expression of *end-1*::GFP is completely absent (Shetty et al., 2005). POP-1 functions in the E cell through the action of Wnt/Src/MAPK pathway provided by a cell-cell interaction between P_2 and EMS (Shetty et al., 2005).

Wnt signaling pathway

The Wnt signaling pathway is one of many conserved signal transduction pathways used widely during animal development, from Hydra to humans (Cadigan and Nusse, 1997; Hobmayer et al., 2000; Peifer and Polakis, 2000; Wodarz and Nusse, 1998). The name 'Wnt' was established by a combination of the gene *Wg* (*wingless*) in *Drosophila* and the mouse mammary oncogene *int-1* (Rijsewijk et al., 1987). The *wingless* gene was originally identified as a recessive mutation that resulted in abnormal development of the wings and haltere (Sharma and Chopra, 1976). The *int-1* (or *Wnt-1*) gene was originally identified as a gene with integration sites for the mouse mammary tumor virus (MMTV), resulting in mammary carcinomas in mice (Nusse et al., 1984). Both *wingless* and *int-1* were found to be homologous with similar amino acid sequences of their encoded proteins (Rijsewijk et al., 1987).

A simple diagram of the canonical Wnt pathway is shown in Figure 8. Wnt proteins that are secreted and presented to the surface of the cells bind to Frizzled (Fz)/low density lipoprotein receptor-related protein (LRP) complex at the cells surface. Upon activation, the receptors send signals to several proteins within the cell which include, Dishevelled (Dsh), glycogen synthase kinase-3β (GSK-3), Axin, Adenomatous Polyposis Coli (APC), and the transcriptional regulator, β-catenin (Logan and Nusse, 2004). Levels of cytoplasmic β-catenin are typically low within the cell as a result of proteasome degradation mediated by a complex of proteins, GSK-3/APC/Axin. However, when the Wnt pathway is activated upon binding of ligand and receptor, the degradation pathway is blocked and results in accumulation of β-catenin in the cytoplasm and nucleus

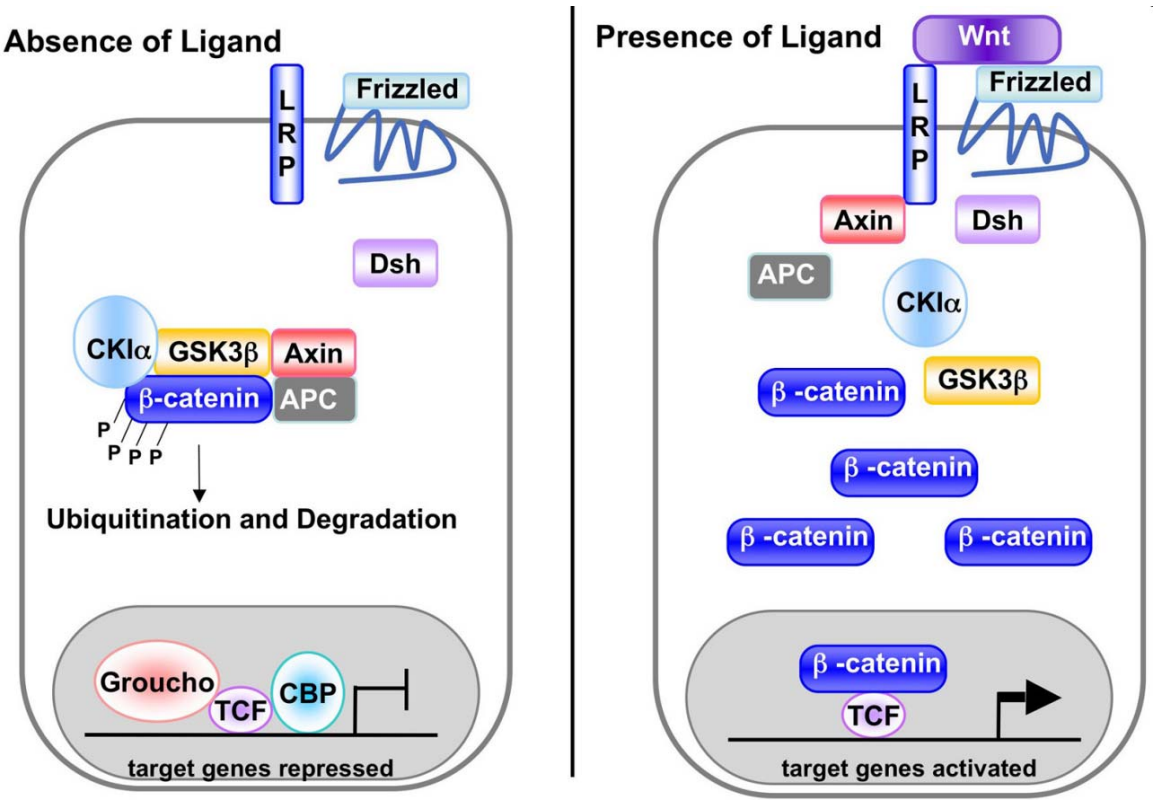


Fig. 8. A canonical Wnt signaling pathway. In the absence of ligand, action of the destruction complex (CKIα, GSK3β, APC, Axin) creates a hyper-phosphorylated β-catenin, which is a target for ubiqitination and degradation by the proteosome. Binding of Wnt ligand to a Frizzled/LRP-5/6 receptor complex leads to stabilization of hypo-phosphorylated β-catenin, which interacts with TCF/LEF proteins in the nucleus to activate transcription. In a canonical pathway, CKIα, GSK3β, APC, and Axin act as negative regulators and all other components act positively (Eisenmann, 2005). Figure composted by Wormbook (Eisenmann, D. M., Wnt signaling (June 25, 2005), *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.7.1, http://www.wormbook.org).

(Logan and Nusse, 2004). Nuclear β-catenin then interacts with transcription factors, lymphoid enhancer-binding factor 1/T cell-specific transcription factor (LEF/TCF) to regulate transcription of numerous target genes, some being members of the Wnt pathway resulting in feedback control (Logan and Nusse, 2004). In the absence of Wnt signal, LEF/TCF factors bind DNA and interact with other transcription factors (i.e., Groucho, histone deacetylase) to repress transcription (Eisenmann, 2005).

Work in *Drosophila* and vertebrates have shown that Wnt signals can be transmitted in two discrete ways: through the well-known Wnt/β-catenin pathway (canonical pathway) or through β-catenin independent (non-canonical) pathways (Eisenmann, 2005). The non-canonical pathway is not as clearly understood as the canonical Wnt pathway. However, studies have shown that non-canonical pathways play an essential role in controlling aspects of gastrulation movements similar to that of *Drosophila* planar cell polarity (PCP) pathway, which controls the orientation of hairs, bristles, and ommatidia (Veeman et al., 2003). The non-canonical pathway has also been indicated to be involved in various processes such as, cochlear hair cell morphology, heart induction, dorso-ventral patterning, tissue separation, neuronal migration, and cancer (Veeman et al., 2003).

In sea urchin embryonic development, endomesoderm specification involves Wnt/β-catenin signaling. Studies have shown that nuclearization of β-catenin is one required input for proper specification of the veg_2 lineage (Oliveri et al., 2002)—cells that give rise to endoderm, the coelom (body wall), and secondary mesenchyme (pigment cells, immunocytes, and muscle cells) (Logan, 1999). In addition, the Wnt8 ligand,

which activates the β-catenin/TCF complex, is also expressed and required for specification of endomesoderm (Davidson et al., 2002). The Wnt signaling pathway is involved in various mammalian developmental processes, as well. These include cell proliferation, differentiation, and epithelial-mesenchymal interactions, which contribute to development of tissues and organs such as the limbs, the brain, the reproductive tract and the kidney (Smalley and Dale, 1999). Any disruption or alterations that occur in the Wnt signaling pathway can be fatal and is a major factor in neoplasia and an oncogenic activation of this pathway can occur at various levels (Smalley and Dale, 1999).

A study has demonstrated a link between the Wnt signaling pathway and regulation of cell proliferation and differentiation present in mice that were null for TCF-4, which binds to stabilized β-catenin and activate transcription. The mice underwent a normal transition of endoderm to intestinal epithelium during embryogenesis, but failed to maintain "proliferative compartments" in the crypts of the small intestine, rather became differentiated, non-dividing cells (Korinek et al., 1998). Whether mutations in TCF lead to activation in cancer cells is unclear, but it has been shown that mutations in TCF-4 have led to colorectal tumors (Duval et al., 1999). The manifestation of cancer by aberrant Wnt signaling, most likely results from inappropriate gene activation mediated by stabilized β-catenin (Morin et al., 1997; Rubinfeld et al., 1997). For example, in many cancers β-catenin is found to be accumulated in the nuclei where it forms complexes with LEF-1 (Henderson et al., 2002). In normal cells, levels of β-catenin are regulated by adenomatous polyposis coli (APC) through nuclear export and degradation. LEF-1, however, functions to anchor β-catenin in the nucleus and block

APC-mediated export (Henderson et al., 2002). In cancer cells such as colon cancer and melanoma, where LEF-1 is over-expressed, β-catenin levels remain high in the nuclei and results in constitutive activation of target genes (Henderson et al., 2002) that can lead to cellular growth, differentiation, and survival (Polakis, 2000).

In most invertebrates and vertebrates, including *Drosophila* and mammals, a single β-catenin regulates both cell adhesion and Wnt signaling (Bienz, 2005). For example, in *Drosophila* a single β-catenin, Armadillo, functions in both Wnt signaling and cell adhesion (Orsulic and Peifer, 1996). In the *Drosophila* embryo, *armadillo* is required to transduce *wingless* during antero-posterior patterning of the larval cuticle and during midgut formation (Peifer et al., 1991; Siegfried et al., 1994). In the *Xenopus* embryo, maternal β-catenin is required for the induction of the dorsal axis (Fagotto et al., 1996), while over-expression of β-catenin induces formation of an ectopic axis (Heasman et al., 1994). In *C. elegans*, however, four β-catenin homologs: HMP-1, BAR-1, WRM-1, and SYS-1, each have different and specific roles in development (Korswagen, 2007). HMP-2 and BAR-1 function in cell adhesion and in the canonical Wnt pathway, respectively, however WRM-1 and SYS-1 are distinctively required in the non-canonical Wnt/MAPK asymmetry pathway, to regulate asymmetric cell division (Korswagen, 2007). The Wnt/MAPK signaling pathway relies on both the components of the canonical Wnt pathway (e.g., frizzled receptor, disheveled, β-catenin, TCF) and the MAPK-related pathway (e.g., MOM-4/TAK-1, also called TGF-β-activated kinase, and LIT-1/NLK, also called nemo-like kinase).

Several of the Wnt/MAPK regulators are required to give rise to POP-1 asymmetry. As previously mentioned, the EMS blastomere divides into two asymmetric daughter cells, MS (anterior daughter cell) and E (posterior daughter cell). The specification of MS and E is a result of regulation via the SKN-1 pathway, as well as the Wnt/MAPK signaling pathway derived from the P_2 and EMS interaction, an example of combinatorial control. This induction has been analyzed in vitro by isolating and culturing blastomeres from early embryos. If EMS is isolated and left to divide and develop on its own, it fails to produce endoderm and two MS-like daughters are specified as a result (Goldstein, 1993). However, if P_2 is placed in contact with EMS early on in development, the EMS daughter born posterior (next to P_2) produces endoderm, whereas the anterior daughter cell adopts an MS-like fate (Goldstein, 1993). In the MS cell, POP-1 forms a complex of repressors to block the endoderm specifying genes (*end-1*and *end-3*). However, due to the presence of the Wnt signal provided by P_2, a β-catenin-like factor, SYS-1, binds to POP-1 in the E cell, blocking its repressive activity and leads to activation of the target genes *end-1* and *end-3* (Thorpe et al., 1997).

Roles of the β-catenin-like factors, WRM-1 and SYS-1, in binary cell fate specification

Upon activation of the Wnt signaling pathway in *C. elegans*, the TCF/LEF-like factor, POP-1 is phosphorylated by a the WRM-1/β-catenin and LIT-1/NLK complex, resulting in POP-1 to be exported out of the nucleus of posterior daughter cell (Rocheleau et al., 1999). In the absence of *wrm-1*, POP-1 localizes symmetrically in the nuclei of both daughter cells resulting in the posterior daughter cell, for example the E blastomere,

to adopt the fate of the anterior daughter cell (i.e., the MS blastomere) (Korswagen, 2002). Here, the function of WRM-1/β-catenin is clearly not the same as a canonical β-catenin, which is a transcriptional co-activator for TCF transcription factors. Rather, it functions to reduce nuclear levels of POP-1 in response to the Wnt/MAPK pathway, in the posterior daughter cell where WRM-1 levels are high (Mizumoto and Sawa, 2007).

However, a different β-catenin transcription factor acts as a co-activator and is responsible for causing POP-1 to function differently in the daughter cells. The *sys-1* gene (*sy*mmetrical *s*isters), was identified in a genetic screen for regulators of gonadogenesis. Hermaphrodites that are homozygous mutants develop severely abnormal gonads and are 100% sterile (Miskowski et al., 2001). In addition, *sys-1* mutants show defects in the fate specification of the posterior daughter of the post-embryonic epidermal T cell and in the transcriptional activation of POP-1 targets in the E blastomere (Huang et al., 2007; Kidd et al., 2005). During asymmetric cell divisions, such as those of the EMS cell, SYS-1 levels increase in the "activated"/posterior daughter cell, while it is absent or detected at very low levels in the "inactive"/anterior daughter cell (Fig. 9). This, in fact, is reciprocal to levels of POP-1 (higher in posterior than in the anterior cells). These observations, as well as others, suggest that the large amount of SYS-1 in the nucleus of the posterior daughter cell ensures that the POP-1 protein, which is of low abundance, binds to SYS-1, converting POP-1 to its transcriptionally active state (Mizumoto and Sawa, 2007). Even though the amino acid sequence of SYS-1 has minimal homology to β-catenin, it can bind POP-1 in the nucleus and activate the transcription of target genes (Kidd et al., 2005). Together, the POP-1—SYS-1 complex

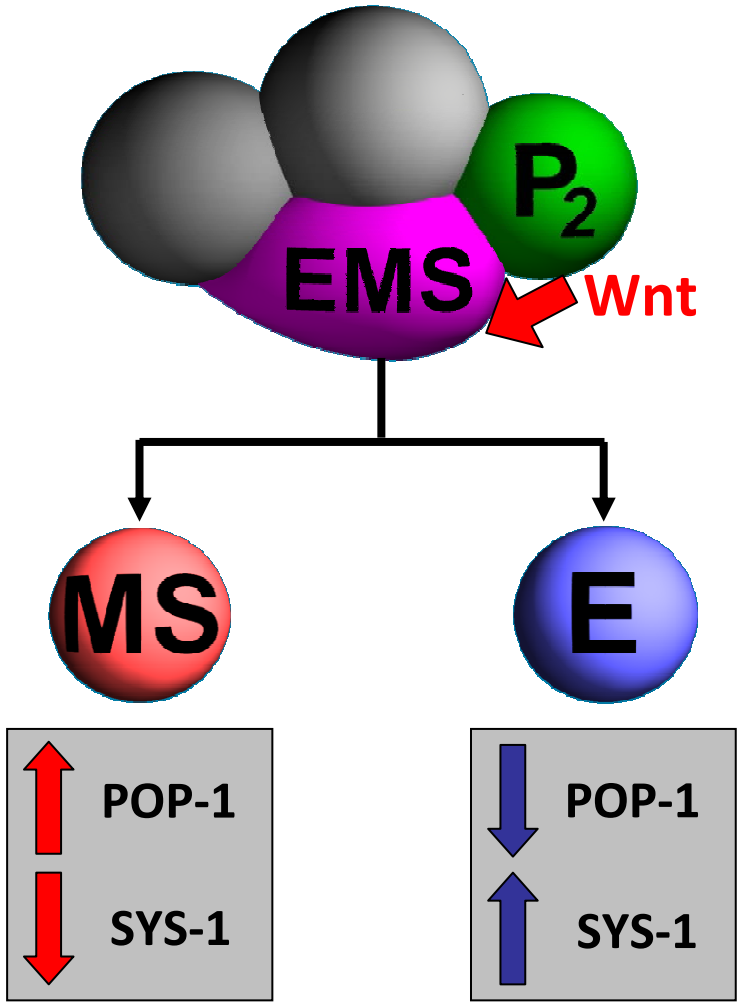


Fig. 9. Wnt/β-catenin signaling pathway results in asymmetric cell division in *C. elegans*. If the EMS blastomere is isolated from the embryo at the 4-cell stage, it will give rise to two MS daughter cells. However, in the intact 4-cell embryo, the P_2 cell sends a Wnt signal to the posterior end of its sister cell, EMS. In the MS cell POP-1 known to act as a repressor of endoderm genes, *end-1*and *end-3*, however upon Wnt signaling in the E blastomere, the inhibitory function of POP-1 is modified for it to become an activator of *end-1,3*. The β-catenin-like factor, SYS-1, acts to bind to TCF/LEF-like factor, POP-1, and activate transcription. In the MS cell, nuclear levels of SYS-1 are low, due to the absence of Wnt signal, and nuclear POP-1 levels are high. However, the presence of Wnt signaling in the E cell results in higher levels of SYS-1 in the nucleus and lower levels of POP-1. This reciprocal expression of β-catenin- and TCF/LEF-like factors is what leads to the asymmetric specification of the EMS daughter cells, MS and E. Part of figure composited by M. Maduro

activates transcription of its direct target, *end-1*, in the posterior daughter of the EMS cell (E blastomere) (Huang et al., 2007), and *ceh-22* in the distal daughters of somatic gonad precursor (SGP) cells (Miskowski et al., 2001), Z1 and Z4 (Lam et al., 2006). Therefore, SYS-1 is known to be a functional β-catenin homolog. In the nucleus of the anterior cell, however, the amount of SYS-1 is lower compared with the high level of POP-1, thus resulting in POP-1 being in the SYS-1-unbound state and, as a result, POP-1 functions as a transcriptional repressor (Mizumoto and Sawa, 2007). In summary, both β-catenin factors function to regulate POP-1 transcriptional activities in two different ways that create reciprocal asymmetry. First, upon activation of the Wnt/MAPK signaling pathway, WRM-1/LIT-1 exports POP-1 in the posterior daughter cell creating lower levels of POP-1 compared to high levels in the anterior cell. Next, the higher levels of SYS-1 in the posterior cell results in its binding to nuclear POP-1 and activating transcription. However, in the anterior cell POP-1 levels are high and SYS-1 levels are low (due to degradation), resulting in POP-1 functioning as a transcriptional repressor. Therefore, reciprocal asymmetries of POP-1 and SYS-1 creates a robust binary fate specification in both daughter cells after cell division (Mizumoto and Sawa, 2007).

The roles of TCF/LEF factor: transcriptional activation and repression

The original members of the TCF family, T cell-factor 1 (TCF-1) and lymphoid enhancer binding factor-1 (LEF-1), were first isolated as lymphoid-specific DNA-binding proteins (Clevers and van de Wetering, 1997). These proteins, as well as their homologues in *Drosophila*, *Xenopus* and *C. elegans*, contain a DNA-binding domain,

called high mobility group (HMG), previously mentioned, as well as a conserved N-terminal domain that binds nuclear β-catenin. In addition, some members of the TCF-1/LEF-1 family contain the POP-1 domain located C-terminally to the HMG box (Waltzer and Bienz, 1999). The first correlation observed between TCF and Wnt signaling was found by yeast two-hybrid screens, which identified β-catenin as a protein binding to mammalian LEF-1 (Behrens et al., 1996) and to *Xenopus* XTcf-3 (Molenaar et al., 1996). As it is demonstrated in *C. elegans* embryonic development, the TCF/LEF transcription factor plays a dual role in which it acts as a repressor, but switches its role as an activator upon binding of β-catenin. As a repressor, TCF bind histone deacetylase (HDACs) (i.e., HDA-1), and co-repressors (i.e., Groucho or CtBP) in the absence of β-catenin (Daniels and Weis, 2005; Hurlstone and Clevers, 2002). Upon activation of the Wnt pathway, β-catenin antagonizes Groucho binding and converts TCF as a repressor to a transcriptional activator by recruiting chromatin remodeling complexes and/or other transcriptional co-activators.

The activity of TCF has been shown to be conserved within several model systems. In *Xenopus* embryonic development a complex variety of XTcf factors are present. Each XTcf member binds either β-catenin and/or Groucho to regulate transcription of target genes, and is ubiquitously expressed throughout the early embryo. XTcf3, for example, binds the co-repressor Groucho (Molenaar et al., 2000; Roose et al., 1998) but also activates the expression of *engrailed* and *siamois*, genes that contain the TCF/LEF binding sites, upon binding of β-catenin (Brannon et al., 1997; McGrew et al., 1999). In addition, XTcf1 has been found to act similarly to XTcf3 where it is a

transcriptional repressor ventrally and an activator dorsally. However, XTcf4 was found to act only as a transcriptional repressor (Standley et al., 2006). Unlike *C. elegans*, *Xenopus* has several TCF-like factors that either act as a transcriptional "switch" (both a repressor and activator; i.e., XTcf1 and XTcf3) or act only as a repressor (i.e., XTcf4). In *Drosophila* embryonic development, signal activity from Wingless/Wnt stabilizes the cytoplasmic β-catenin-like factor Armadillo (Cadigan and Nusse, 1997), which forms a bipartite transcription factor with the HMG-box protein *Drosophila* Tcf (dTcf) and activates expression of Wingless target genes (Brunner et al., 1997; Riese et al., 1997; van de Wetering et al., 1997). However in the absence of Wingless signaling, Armadillo is not stabilized and dTcf acts as a transcriptional repressor, in addition to Groucho (Gro) binding as a co-repressor (Cavallo et al., 1998). Grg5, which is a transcriptional co-repressor related to the *Drosophila* Groucho, has also been identified as an additional TCF binding partner. Grg5 protein interacts with histone deacetylase -1 (HDAC) and mediates condensation of the chromatin (Chen et al., 1999). Collectively, these studies demonstrate the conserved dual role of the TCF transcription factor throughout development of several model systems.

The Notch/Delta signaling pathway

In addition to the Wnt signaling pathway, the Notch signaling pathway has been conserved within metazoans and functions in cell fate determination through cell-cell interactions. The gene encoding the Notch transmembrane receptor was discovered about 80 years ago in *Drosophila melanogaster* through a partial loss-of-function mutation that

resulted in “notches” at the wing margin (Mohr, 1919). Notch-like proteins have been identified in *C. elegans* (LIN-12 and GLP-1) (Greenwald, 1998; Kimble and Simpson, 1997), sea urchins, and many different vertebrates, including humans (Gridley, 1997; Sherwood and McClay, 1997). In all animal models tested, mutations in the Notch receptor resulted in aberrant developmental characteristics, as well as human pathologies (Joutel et al., 1996; Li et al., 1997). Many studies have led to identifying several proteins involved in transmitting or regulating the signals received by Notch. In *Drosophila*, two transmembrane proteins, Delta and Serrate, have been characterized as redundant Notch ligands (Delta and Jagged in vertebrates; LAG-2 and APX-1 in *C. elegans*) (Greenwald, 1998; Gridley, 1997; Gu et al., 1995). A major downstream effector of Notch signaling is the transcription factor Suppressor of Hairless [Su(H)] (CBF/RJBk in mammals; LAG-1 in *C. elegans*), which leads to the regulation of Notch target genes *Enhancer of split* [*E(spl)*] that encode the nuclear basic helix-loop-helix (bHLH) proteins (Egan et al., 1998; Greenwald, 1998) (Fig. 10 A). In summary, activation of the signaling pathway occurs when an interaction forms between the Notch receptor and the DSL (Delta/Serrate/LAG-2) ligand family. Upon activation a cleavage event takes place in which the intracellular portion of the Notch receptor (ICN) is released and translocates to the nucleus to activate transcription of target genes upon interaction with another transcription factor, LAG-1/Su(H). In some instances, lateral signaling takes place in which two or more equivalent cells express both ligand and receptor. The interaction between the two cells will result in modulation of receptor/ligand levels to achieve asymmetry or different cell fates (Wilkinson et al., 1994).

During the first four cleavages in the *C. elegans* embryo, GLP-1 functions in two known cell-cell interactions (Hutter and Schnabel, 1994; Mello et al., 1994; Priess et al., 1987). The first interaction occurs during the 4-cell stage where GLP-1 is expressed by two equivalent daughter cells of AB (ABa and ABp) (Crittenden et al., 1994) and the ligand, APX-1, expressed by P_2, where P_2 induces ABp to adopt a fate different from ABa (Mango et al., 1994; Mickey et al., 1996) (Fig. 10 B). In *apx-1* mutant embryos, ectopic expression of pharyngeal tissue results (Mello et al., 1994), whereas absence of *glp-1* results in no anterior pharynx made (Hutter and Schnabel, 1994). Collectively, these results led to the conclusion that the interaction between ABp (expressing GLP-1 receptor) and P_2 (expressing APX-1 ligand) is essential in that P_2 signals ABp to not make pharynx tissue (Hutter and Schnabel, 1994; Mello et al., 1994). In addition, GLP-1, localized on the membrane of ABa, receives a signal from MS to make anterior pharynx at the 12-cell stage (Mello et al., 1994) (Fig. 10 B). Overall, the GLP-1/Notch induced signaling that occurs in the early embryo results in the establishment of the anterior/posterior axis of the AB blastomere, as well as establishment of asymmetry along the left/right axis (Hutter and Schnabel, 1994). The second GLP-1 interaction occurs during post-embryonic development in the germline, where GLP-1 is expressed and activated by the ligand, LAG-2, produced by a somatic gonadal cell, to promote mitosis (Crittenden et al., 1994; Henderson et al., 1994).

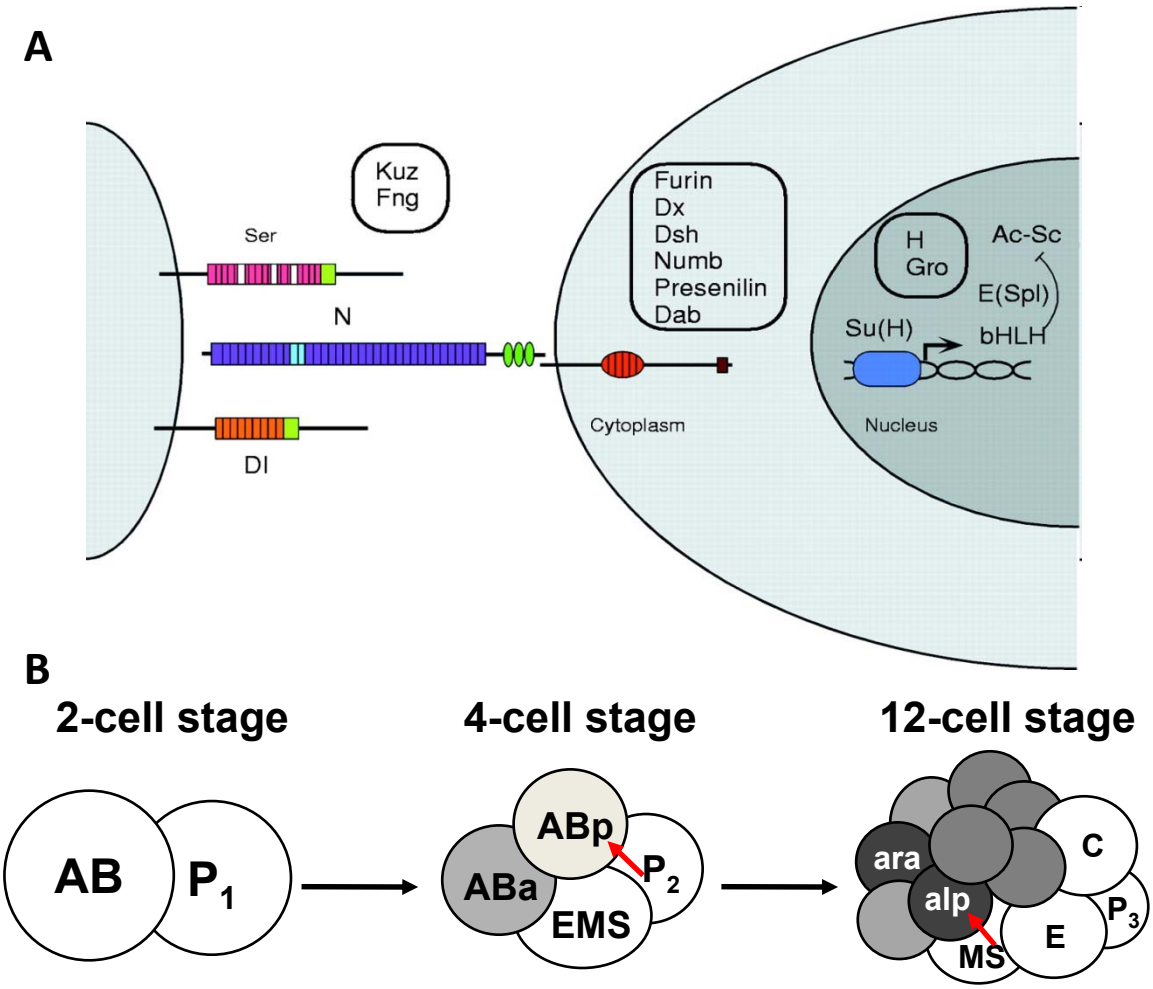


Fig. 10. (A) Elements of Notch signaling. Extracellular regions of Notch (N) and Delta (Dl) interact to activate the receptor. As a result of activation, the Supressor of Hairless [Su(H)] transcription factor binds to regulatory sequences of the *Enhancer of split* [E(Spl)] complex genes, which encode bHLH proteins. bHLH products, together with Groucho, can repress the expression of the *Achaete-Scute* (Ac-Sc) proneural genes (Artavanis-Tsakonas, 1999). **(B) Notch signaling in *C. elegans* early embryo.** In *C. elegans* the Notch-like receptor, GLP-1, is involved in two cell-cell interactions. The first interaction occurs at the 4-cell stage in which, GLP-1, expressed by the two AB cells, interacts with the Delta-like ligand, APX-1, localized on the membrane of the P_2 cell. The P_2 and ABp interaction causes ABp to not make anterior pharynx. The second GLP-1-mediated induction occurs at the 12-cell stage in which MS sends the signal to the descendants of ABa (ABara and ABalp), resulting in anterior pharynx to be made (Hutter and Schnabel, 1994; Mello, et al., 1994).

Goal of thesis: To elucidate the role of zygotic genes, *tbx-35* and *ceh-51*, and the maternal gene *pop-1* in specification of MS blastomere and tissue identities in the early *C. elegans* embryo

As previously described, maternal gene products have been well-characterized and shown to have a major contribution in establishing the cell fate of the early *C. elegans* embryo. Two maternal pathways, SKN-1 pathway and the Wnt/MAPK signaling pathway, have been shown to work together to activate downstream zygotic regulators (i.e., *med-1,2*, *tbx-35*, *end-1,3*, etc). However, research is still underway in determining how later zygotic genes (i.e., *tbx-35*) contribute to the specification of tissue identity and how they fit in to the later parts of the gene regulatory network. Although, *tbx-35(-)* embryos lack most tissues made by MS (Broitman-Maduro et al., 2006), it was not known what accounted for the much stronger loss of MS tissues in a *med-1,2(-)* double mutant. For example, it was found that *tbx-35* is necessary for specification of early MS-derived tissues, such as posterior pharynx and body wall muscle (tissues develop within 5-6 hours into embryogenesis), however about two hours after those tissues are made, the coelomocytes are born. As a marker of a minor tissue type made by MS that is specified much later in embryonic development, we were interested to determine if the MS-blastomere identity gene, *tbx-35*, plays a role in specification of such tissues, as well.

Previous studies have shown that loss of maternal *pop-1* activity results in expression of *end-1* and *end-3* in MS, as well as a MS to E-like transformation (Maduro et al., 2007; Maduro et al., 2005b). However, *tbx-35* also continues to be expressed in MS, even in the absence of *pop-1* (Broitman-Maduro et al., 2006). In addition, recent

findings in our lab have shown that in the closely related nematode *Caenorhabditis briggsae*, POP-1 functions differently in that it contributes to MS and E specification, specifically acting as an activator of *Cb-end* genes in E and works in parallel to specify the MS fate with *Cb-skn-1* (Lin et al., 2009). These findings led to the question of whether *C. elegans* POP-1 may function in a different way besides repression of endoderm fate in the MS blastomere. Taken together, I was interested to further characterize the endomesoderm gene regulatory network by probing the question of a possible, additional role of *pop-1* in *C. elegans* early embryo and identifying factors required to specify later MS-derived tissues such as coelomocytes. The results obtained throughout my thesis provide an added insight to how a complex network of genes and signaling pathways interconnect to give rise to specified fates in the early *C. elegans* embryo. The findings may offer answers for the embryonic development of more complex organisms such as mammals, particularly humans. Although, nematodes are far simpler, surprisingly they share similar genes and protein functions (i.e., T-box family of genes, Wnt signaling, etc.) with higher organisms (Dierick and Bejsovec, 1999; Ryan and Chin, 2003; Wattler et al., 1998). Novel findings in embryonic development in simple organisms such as nematodes can help researchers reach the first step in finding a treatment for genetically pre-disposed diseases or possibly cancer.

Chapter 2: Materials and Methods

Strains used

Strain	Mutations	Transgenes	Reporter
NP749		*cdIs42 I* [*cup-4*::GFP]	*cup-4*::GFP
MS862*†	*tbx-35(tm1789)*	*cdIs42 I*; *irEx274* [*tbx-35(+)* and *rol-6D* and *unc-119*::CFP]	*cup-4*::GFP
MS1268	*ceh-51(tm2123)*	*cdIs42 I; irEx544* [*Y80D3A.3(+), unc-119*::mCherry]	*cup-4*::GFP
MS887	*med-1(ok804); med-2(cx9744)*	*cdIs42 I*; *irEx138* [*med-1(+)* and *unc-119*::CFP]	*cup-4*::GFP
MS884		*cdIs42 I*; *irIs42 X* [*hs-tbx-35*]	*cup-4*::GFP
MS897	*end-3(ok1448)*	*cdIs42 I*?	*cup-4*::GFP
MS934		*wIs88 ?* [*hs-med-1*]; *cdIs41*	*cup-4*::GFP
MS928		*cdIs41*; *irIs57* [*hs-ceh-51*]	*cup-4*::GFP
MS972	*tbx-35(tm1789); end-3(ok1448)*	*cdIs42*; *irEx274*	*cup-4*::GFP
MS1093	*end-1(ok558); end-3(ok1448)*	*cdIs42*; *irEx498* [*unc-119*::RFP, *end-3(+)*]	*cup-4*::GFP
MS1359	*end-1(ok558); end-3(zu247)*	*cdIs42*; *irEx568* [*sur-5*::dsRed rescue array]	*cup-4*::GFP
MS1109	*med-1(ok804); med-2(cx9744); end-1(ok558); end-3(ok1448)*	*cdIs42*; *irEx502* [*unc-119*::mCherry resc. *med-1(+)* and *end-3(+)*]	*cup-4*::GFP
MS1301	*tbx-35(tm1789); ceh-51(tm2123)*	*cdIs42*; *irEx570* [*unc-119*::mCherry resc. *tbx-35(+), ceh-51(+)*]	*cup-4*::GFP
MS1332	*tbx-35(tm1789); ceh-51(tm2123); end-1(ok558); end-3(ok1448)*	*cdIs42*; *irEx570* [*unc-119*::mCherry resc. *tbx-35(+), ceh-51(+)*]; *wEx1506* [*end-3(+), unc-119(+), unc-119*::YFP]	*cup-4*::GFP
SM469		*pxIs6*	*pha-4*::GFP
MS1000	*end-3(ok1448); glp-1(or178)*	*pxIs6*	*pha-4*::GFP
MS1101	*end-1(ok558); end-3(ok1448); glp-1(or178)*	*pxIs6; wEx1506*	*pha-4*::GFP
MS1075	*end-1(ok558); end-3(ok1448)*	*pxIs6; irEx490* [*unc-119*::CFP *and end-3(+)*]	*pha-4*::GFP
MS1369	*end-1(ok558); end-3(zu247)*	*pxIs6; irEx568* [dsRed resc. array]	*pha-4*::GFP

MS1097	*med-1(ok804); med-2(cx9744); end-1(ok558); end-3(ok1448)*	*pxIs6; wEx1506; irEx138* [*med-1(+), unc-119*::CFP]	*pha-4*::GFP
MS929	*med-1(ok804); med-2(cx9744)*	*pxIs6; irEx138*	*pha-4*::GFP
MS1303	*tbx-35(tm1789); ceh-51(tm2123)*	*pxIs6; irEx295* [*tbx-35(+), unc-119*::YFP]*; irEx540* [*ceh-51(+), unc-119*::CFP]	*pha-4*::GFP
MS1370	*tbx-35(tm1789); ceh-51(tm2123); end-1(ok558); end-3(ok1448)*	*pxIs6; irEx570; wEx1506*	*pha-4*::GFP
AZ217		*ruIs37*	*myo-2*::GFP
MS1088	*end-1(ok558); end-3(ok1448)*	*ruIs37; wEx1506*	*myo-2*::GFP
MS739‡	*pop-1(zu189)*	*ruIs37* [*myo-2*::GFP and *unc119(+)*]	*myo-2*::GFP
MS758‡	*pop-1(zu189); end-3(ok1448)*	*ruIs37*	*myo-2*::GFP
MS1196‡†	*end-1(ok558); end-3(ok1448); dpy-5 (e61) pop-1(zu189)*	*ruIs37; irEx524* [*unc-119*::CFP]	*myo-2*::GFP
KM402		*gvIs402*	*unc-120*::GFP
MS1080	*end-1(ok558); end-3(ok1448)*	*gvIs402; wEx1506*	*unc-120*::GFP
HC395		*qtIs9*	*nhr-25*::YFP
MS1250	*end-1(ok558); end-3(ok1448)*	*qtIs9; irEx568*	*nhr-25*::YFP
MS1338	*tbx-35(tm1789); ceh-51(tm2123)*	*qtIs9; irEx577* [*tbx-35(+), ceh-51(+), sur-5*::dsRed]	*nhr-25*::YFP
MS1371	*end-1(ok558); end-3(ok1448)*	*qtIs9; cdIs42; irEx568*	

* The *tbx-35(tm1789); unc-119*::CFP strain, MS516 (Coroian et al., 2006), was crossed with the NP749 strain, resulting in generation of the following genotype, *tbx-35*; *unc-119*::CFP; *cup-4*::GFP, named MS862.
† Strains constructed by myself. Others constructed by M. Maduro.
‡ *pop-1(zu189)* strains were balanced by a reciprocal translocation, hT1.

Worm handling

<u>*Maintenance*</u>: Worms were cultured on NGM (Nematode Growth Medium) agar plates of various sizes over a lawn of *E. coli* OP50, which is used as their food source in the laboratory (Brenner, 1974). *E. coli* OP50 is a uracil auxotroph whose growth is limited on

agar plates. A limited bacterial lawn is desirable because it allows for easier observation and better mating of the worms. Several sizes of petri plates are available and can be purchased from companies such as Falcon. Smaller plates (35 mm diameter) are useful for matings/crosses or when using expensive drugs. Medium size plates (60 mm diameter) are useful for general strain maintenance, and larger plates (100 mm diameter) are useful for growing larger quantities of worms, such as for certain mutant screens . The NGM plates (Stiernagle, 2006) can be poured into petri plates easily and using a peristaltic pump(Stiernagle, 2006). This pump can be adjusted so that a constant amount of NGM is dispensed into each petri plate, which is necessary in order to reduce the need for refocusing the microscope when you switch from one plate to another (Stiernagle, 2006). It is essential to not allow the worms to starve in culture. The worms will go into the dauer stage, in which animals at the L1 stage will arrest in the molting process until environmental conditions are optimum (Hu, 2007). Conditions such as low food supply, ambient temperature, and high population density can result in animals going into the dauer stage (Hu, 2007). To prevent starvation or to allow worms to come out of the dauer stage and continue with developmental process, worms were transferred onto a freshly, seeded large or medium NGM plate.

Experiment preparation: When RNAi experiments, laser ablation studies, or any other technique that required a new, synchronized generation of animals, a dense plate of adult, gravid worms (worms carrying developing eggs in uterus) were rinsed with M9 and transferred into several 1.5 mL eppendorf tube or 15 mL falcon tube, depending on

amount/concentration of worms. Typically, a plate or two was transferred with 2 ml of saline solution, M9 (Stiernagle, 2006), into two separate Eppendorf tubes. Filled tubes would be closed with the cap tightly and placed into a table-top micro-centrifuge and spun for up to 30 seconds. M9 solution was removed from worm pellets and 1.5 mL of bleach was dispensed into each tube. Tubes would be shaken gently for approximately 5 minutes or until adult worms have dissolved, leaving only embryos in solution. Careful observation was taken under light microscope to ensure that embryos did not dissolve as a result of prolonged exposure to the bleach. Once the worm carcasses were no longer visible in bleach solution, the tube(s) were centrifuged for about 30 seconds to pellet embryos. Pelleted embryos were rinsed thoroughly, three to four times, with 1 mL of M9 to get rid of remaining bleach. After rinsing, embryos were transferred to 8 mL of M9 and 8 μL of cholesterol solution and placed in 20°C or 15°C incubator until embryos hatched and became synchronized at the developmental stage. Thus young, synchronized worms were placed on an NGM plates for experimentation (RNAi, laser ablations, etc.).

RNA interference (RNAi) experiments

As previously described, Fire and Mello discovered that injection of double stranded RNA (dsRNA) into worms leads to specific degradation of the corresponding mRNA, (RNA interference; RNAi) (Fire et al., 1998). Several methods of administering dsRNA to worms included, feeding (Timmons and Fire, 1998) and injection (Fire et al., 1998), but soaking (Tabara et al., 1998) is a method used, as well. All three can result in efficient gene knockdown, although the use of a technique depends on the experiment.

Majority of gene knockdown methods used in the experiments described below were carried out through feeding dsRNA.

As an initial step to engineer dsRNA of a particular gene of interest, the coding region of the gene was amplified via PCR and cloned into pPD129.36. Then, ligation and transformation were followed using *E. coli* competent cells (XL-2 Blue). The transformation was done with *E. coli* competent cells first, in order to select for recombinant plasmids. Once the clone was successfully obtained, another transformation step followed using *E. coli* HT115 cells (Timmons and Fire, 1998). The HT115 bacterial cells containing the gene of interest were used to obtain dsRNA and fed to worms. L4 animals were fed for ~36 hours on agar feeding plates containing 1 mM IPTG and 25 μg/ml Carbenicillin seeded with HT115 bacteria containing dsRNA of interest. Adults were transferred to fresh plates (same plates used to maintain animals) for egg-laying for 4-6 hours at 20ºC. Adults were removed, leaving only the eggs, in order to score embryos that have developed in the same time interval. Embryos were allowed to develop for ~12-16 hours prior to scoring.

Laser ablations, microscopy, and imaging

Laser ablations: Laser ablation studies have been conducted on early embryos ranging between the 2- to 8-cell stages. In order to isolate such early embryos, adult, gravid worms were placed in a dissecting dish containing ~10 μL of egg salts (Shaham (ed.)). A 26 gauge ½" needle was used to cut gravid adults. Early embryos that were separated from adult worms were mouth-pipetted, using 100 μL micro-capillary pipettes (Kimble

Glass Inc.), on to an agar pad placed over a microscope slide. The agar pad was prepared using a glass tube mixed with 0.2 grams of agar (Bacto-agar, Difco Laboratories) in 4-5 mL of egg salts. Solution in glass tube was placed over flame and shaken simultaneously to dissolve agar into solution. Once the agar was in solution, the glass tube was placed in a heating block until ready for use. A coverslip was placed over embryos on the agar pad and Vaseline was used to seal the edges of the coverslip, leaving a small opening to fill the space with egg salts, preventing the embryos from desiccating. Cell ablations were performed using a Photonic Microsystems Pulsed Laser on a Zeiss Axioplan at the Core Instrument Facility at UC Riverside. Ablated embryos were then placed in a humid chamber, to allow embryos to grow in a moist, temperate environment, and were analyzed ~8 hours later with an Olympus BX-61 fluorescence microscope. Nomarski (differential interference contrast, DIC) and fluorescent images were taken using a Canon 350D camera. Embryos expressing a GFP reporter were observed using a GFP or YFP filter set and to check whether or not embryos were rescued for the mutant phenotype, via rescue array, CFP or RFP filters were used.

Phalloidin staining: To stain for actin filaments in pharynx muscle, phalloidin staining was done following the protocol obtained from WormMethods (Shaham (ed.), ; Strome, 1986). For staining, L3-L4 animals were rinsed off plate with M9 and transferred to 1.5 mL Eppendorf tube. The tube was placed in a table-top micro-centrifuge and spun for about 30 seconds to pellet worms to the bottom. Liquid was removed and 1 mL of M9 was placed in tube, again, as part of the rinsing process. This step was repeated ~2-3

times to completely remove bacteria. If the pellet of worms were heavily dense, 10 µL of worms were aliquot into a separate Eppendorf tube and froze them, very briefly, into liquid nitrogen. Immediately frozen worms were placed into a speed vac (Savant) on a "high" setting for about 5-10 minutes, or until pellet became completely dry. Next, 10 µL of ice cold acetone was added (for fixation) and placed on ice for ~5 minutes. Acetone was carefully removed and was air dried (~5 min.). In a separate Eppendorf tube 10 µL of 2 U fluorescein-conjugated phalloidin (Molecular Probes) was added and placed in speed vac for 5-10 minutes. 20 µL of M9 was added to dissolve phalloidin and mixture was added to dried worms. Worms were left at room temperature for 0.5-1 hour to allow for proper staining. Afterwards, stained worms were thoroughly rinsed 2-3 times with 1 mL of 1X PBS + Tween. Finally, 5 µL of stained worms was aliquoted and 1 drop of mounting medium containing DAPI was added. Stained worms were placed on microscope slide, covered with coverslip, and edges were sealed.

In situ hybridization: In situ hybridization experiments were conducted by Associate Specialist, Gina Broitman-Maduro and the following protocol was used: (http://www.faculty.ucr.edu/~mmaduro/inSitu/InSituProtocol%28MaduroLab%29-v1.0.pdf)

Cloning

The following is a list of clones constructed that pertain to experiments described in the subsequent chapters:

Plasmid	Content	Primers	Restriction sites	Insert/ Vector/ Total length
pMO2	*mex-3* genomic fragment	F5'-ACCGGCCTTCTAGATAACGTCGAGGCTGCC-3' B5'-AAGCGAAAGCTTGGTTCCCCAGTAGTCGTAGG-3'	*Xba*I/ *Hind*III	500bp/3kb/ 3.5kb
pMO3	*mex-3* dsRNA feeding vector	N/A	*Xba*I/ *Hind*III	500bp/3kb/ 3.5kb
pMO8	*tbx-33* dsRNA feeding vector	N/A	*Spe*I/ *Sac*II	1.4kb/3.4kb/ 4.4 kb
pMO11	*tbx-41*:: pWH317 (long promoter; version a)	F5'-GTACGGATCCATCCATTTTATCTCAATCTTGTATAGTTTC-3' B5'-CTTGGAGCTCACGAGACCATGGACTTCATAATGGAGCTTGG-3'	*BamH*I/ *Sac*I	1.2kb/3kb/ 4.2 kb
pMO13	*tbx-41*:: pWH317 (long promoter; version b)	F5'-GCCCGGATCCATCATCACGCAATCATTTTCAGGCTGTTGA-3' B5'-CTTGGAGCTCACGAGACCATGGACTTCATAATGGAGCTTGG-3'	*BamH*I/ *Sac*I	1.2kb/3kb/ 4.2 kb
pMO15	*tbx-41*:: pWH317 (short Promoter)	F5'-CTGTGGATCCTCTACTGTAGTAGTACTGTAGGGTGTTAGA-3' B5'- CTTGGAGCTCACGAGACCATGGACTTCATAATGGAGCTTGG-3'	*BamH*I/ *Sac*I	531bp/3kb/ 3.5kb
pMO16	*unc-37* dsRNA feeding vector	N/A	*BamH*I/ *Sac*I	1.2kb/3kb/ 4.2kb
pMO20	*hda-1* dsRNA feeding vector	N/A	*BamH*I/ *Sac*I	1.2kb/3kb/ 4.2kb
pMO27	*Ce-ceh-51* (promoter) genomic fragment	F5'-CAATTCCGGCAACTTGCAAATTTGCCGGAA-3' B5'-AAAAGAATTCTTTGTGTGGAATGAATTGGGGGAATTA-3'	*Hind*III/ *EcoR*I	300bp/3.5kb/ 3.8kb
pMO28	GFP:: pMO27	F5'-AAAAGAATTCATGAGTAAAGGAGAAGAACTTTTCACT-3' B5'-AAAAACTAGTTTTGTATAGTTCATCCATGCCATGTGT-3'	*EcoR*I/ *Spe*I	1kb/4kb/5kb
pMO29	*Ce-ceh51* coding I:: pMO28	F5'-AAAAACTAGTATGTCATCATCCAACTATAATATTAAT-3' B5'-AATTGCGGCCGCTGCTAATTGCGAAGAAGA-3'	*Spe*I/ *Not*I	1.3kb/5kb/ 6.3kb
pMO30	*Ce-ceh51* coding II + UTR:: pMO29	F5'-AGCAGCGGCCGCAATTCCAAATTTAGGCAA-3' B5'-TAGTCCGCGGACTTTAAAATACCTAAATGA-3'	*Not*I/ *Sac*II	1.3kb/6.3kb/ 7.3kb

Other clones and plasmids described in the text and not listed on the table above were constructed by others in the lab and references pertaining to the clones are cited in the text.

The procedure described below was followed to design the plasmids listed above:

1. Polymerase chain reaction (PCR) is the first step used to amplify the gene fragment of interest. The oligonucleotides flank the region of the gene of interest and were used in the PCR reaction. In addition, dNTPs, genomic template, 10x buffer and $MgCl_2$, and Taq polymerase were added. The total volume of the PCR reaction had to be 25 µL, therefore ddH_2O was added to reach the total volume.

    a) PCR reactions were placed in a PCR thermocycler (MJ Research, PTC-200) and set the conditions (temperature and time) for each parameter. The PCR cycle included the following general parameters (settings used to amplify 1 kb fragment or less) :

        i. Initial @ 94ºC for 3 mins
        ii. Denature @ 94ºC for 30 sec
        iii. Annealing @ 55ºC for 30 sec
        iv. Synthesis @ 72ºC for 30 sec
        v. Terminal @ 72ºC for 10 mins
        vi. Storage @ 4ºC forever

    The annealing temperature varied depending on the GC content of the oligonucleotides and also depending on the length of the region of the gene being amplified. If the region is greater than 1.5 kb then the time setting for annealing and synthesis increased; typically, the time of synthesis would be increased to 30 seconds for every 1 kb. This also depended on the type of Taq polymerase that was being used in the reaction.

b) Completed reactions and 1 kb DNA ladder (Fermentas) were loaded into 0.7% agarose gel containing 0.5 μL propidium iodide and gel was placed in gel box running the samples at 74-82 volts.

c) After the samples ran long enough (20-30 mins) the DNA fragments were visualized with an ultraviolet transilluminator (Spectroline) and checked with DNA ladder for the correct fragment size. If the band was shown at the expected size, the gel fragment was cut out with a razor blade and placed in an Eppendorf tube to purify DNA with Qiagen Gel Extraction kit (procedure provided in kit for gel extraction was followed).

d) If the PCR product is an insert it would be cloned into a vector. The PCR product also have restriction sites (which were designed to be present during the time oligonucleotides were designed and ordered). Therefore, once the PCR product is purified from the gel, it would be incubated with corresponding restriction enzymes (usually 0.5-1 μL each) at 37ºC for at least three hours or overnight. Once this was complete, the digested PCR product was purified again using the Qiagen DNA purification kit. During this time, the vector in which the insert will be cloned into would be digested as well (with the same restriction enzymes). The digested vector would be loaded on a gel and the largest band (check for expected size) was cut out and purified using gel extraction kit (Qiagen).

e) Once the PCR product and the vector were purified the concentration of the DNA using a spectrophotometer (Nanodrop ND-1000, UCR core facility) was

checked. It is preferred that the concentration of the insert be 80 ng/µL and the vector be 10 ng/µL.

2. The ligation reaction was the next step in which the insert and vector were combined into a reaction containing 5X ligase buffer, and DNA ligase. The final volume is 10 µL therefore, if necessary, ddH_20 would be added to reach the final volume. Also, the amount of insert and vector put into the reaction depended on the concentration, which was measured earlier. As a control, a ligation mixture was prepared that consisted of vector alone (no insert), to ensure that the vector does not ligate with itself. Once all components of the reaction were mixed into an Eppendorf tube, it was placed at 22ºC for 30 mins-1 hr.
3. Transformation was the next step, in which competent bacterial cells uptake the plasmid (which hopefully formed during the ligation process) and as the population of bacterial cells increase the amount of plasmid increases, as well.
    a) A tube of competent cells (i.e., XL-2 *E. coli*) was removed from the -80ºC freezer and thawed on ice. Note: cells need to remain chilled to ensure high transformation efficiency; do NOT vortex cells or thaw with hands.
    b) Once the cells have thawed, 100 µL of cells would be transferred to an Eppendorf tube and would be mixed with 1-2 µL of ligation reaction. Each Eppendorf tube was labeled corresponding to each ligation mixture; and empty tubes were placed on ice.
    c) Tubes were tapped gently, to mix, and placed on ice for 15 minutes.

d) The cells were heat-shocked in a 42ºC water bath for 90 seconds. Cells were not to be shaken.

e) Cells were placed back on ice for 2 minutes.

f) 500 µL of LB was added to each of the cells. The tube was inverted to mix

g) Cells were incubated in a 37ºC water bath for 45 minutes.

h) Cells were spun in a table-top centrifuge at maximum speed (14,000 rpm) for 1 minute.

i) Supernatant was decanted and left ~20-50 µL of sup to resuspend and pipette cells onto plates.

j) Stir rod was sterilized with ethanol and flame; was cooled; cells were pipetted on to LB/carbenicillin plates and spread with stir rod.

k) Plates were incubated in a 37ºC oven overnight (14-18 hrs).

4. Plates were checked for colonies the following day. Control plate with vector alone would have very few, if any, colonies. However, cells that contained both vector and insert on plate would have many colonies. Several colonies were picked with a pipette tip and placed into 5 mL of LB liquid mixed with 5 µL of carbenicillin. Cultures were placed in a shaker set at 37ºC overnight.
5. LB liquid was removed from culture tubes the following day (culture should be cloudy) and spun at about 30,000 rpm in centrifuge for 5-10 minutes. Supernatant was decanted and cells at the bottom of tube were purified with plasmid purification kit (Fermentas, GeneJet plasmid purification kit).

6. Plasmids were digested with corresponding restriction enzymes and loaded onto an electrophoresis gel to check for recombinants.

Heat-shock experiments

Embryos that were still contained in hermaphrodite mothers were heat-shocked for 30-45 minutes at 33ºC (one to two 100 mm plates of gravid worms placed in oven). Plates were removed from 33ºC incubator and settled at room temperature for about 30 minutes. Gravid worms were then bleached to retrieve embryos that have been heat-shocked, approximately at the same time interval of embryonic development (0-3 hours). Embryos were transferred into M9 and cholesterol (liquid culture) until the next morning for observation. A second method used during a heat-shock experiment was approximately 30-40 gravid hermaphrodites were picked from plate(s) and transferred to a fresh plate, then placed in the 33ºC incubator for ~45 minutes. Plate was removed and placed in 20ºC to allow for animals to lay eggs for three hours. Adults are then removed from the plate and terminal embryos were observed under fluorescence microscope the following day. Prior to observations, terminal embryos develop for 6-12 hours, at 20ºC, to allow for proper analysis.

Chapter 3: Identification and characterization of CEH-51, a transcription factor that works with TBX-35 to specify MS

T-Box genes in development

The T-box genes are present in all metazoans and function in a variety of aspects in development ranging from early embryonic cell fate decisions, regulation of the development of extra-embryonic structures, embryonic patterning, and organogenesis (Naiche et al., 2005). The T-box genes encode a family of transcription factors sharing a common sequence in the DNA-binding domain and the first member of the T-box family, *Brachyury*, was discovered from mice in 1927 (Dobrovolskaia-Zavadaskaa, 1927); cloned and sequenced in 1990 (Herrmann et al., 1990). *Brachyury* was discovered through a heterozygous mutation that caused a short tail and embryonic lethality in homozygous mutants (Dobrovolskaia-Zavadaskaa, 1927). T-box genes have been found to play essential roles in development. More specifically, most of the T-box genes are found to be expressed in mesoderm precursor cells at the time of gastrulation. In addition, *Brachyury*, *Xenopus-VegT* (Horb and Thomsen, 1997), and *mouse-Tbx6* (Chapman and Papaioannou, 1998) have been shown to play a role in mesoderm induction and development.

T-box factors and mesoderm development in C. elegans

In the *Caenorhabditis elegans* genome, twenty T-box genes have been identified, more than what has been found in the mammalian genome (Minguillon and Logan, 2003). We have previously reported that the T-box gene, *tbx-35*, is required for

specification of most tissues derived from MS descendants, specifically the body wall muscle and the posterior pharynx (Broitman-Maduro et al., 2006). As previously described, coelomocytes are also specified by the MS blastomere, four of which are derived embryonically and two specified post-embryonically by the M blast cell (a total of six coelomocytes in the adult hermaphrodite). Unlike MS-derived pharynx and body wall muscle which are born as early as 300 minutes after the first cleavage, coelomocytes are born later at about 500 minutes. Although *tbx-35* is necessary for specification of MS-derived pharynx and body muscle in majority of embryos, it was not known whether it is required for making coelomocytes. Coelomocytes are only made in the MS lineage, whereas pharynx is made by the MS and AB lineage. In addition, body wall muscle is made by MS, AB, C, and D lineage. Since coelomocytes are a specific readout for MS specification, it was of interest to further characterize the requirement of *tbx-35* in MS specification using a coelomocyte marker.

A strain expressing *cup-4*::GFP (***c***oelomocyte ***up***take), called NP749, was provided by the Fares laboratory (Patton et al., 2005). With the use of this strain it was first essential to confirm that the *cup-4*::GFP marker is expressed in the right number of cells and in the right place during embryonic development. To confirm expression of the *cup-4*::GFP marker, coelomocytes were scored in wild-type embryos. These embryos contained an average of 3.7 ± 0.2 coelomocytes (n = 105), which is fairly close to the known four embryonically-derived coelomocytes (Fig. 11). The number of coelomocytes counted in these wild-type embryos varied from 0 to 6, while ~60% (63/105) of the embryos contained four coelomocytes. When scoring for GFP-expressing cells, the

numbers may vary at times, since the reporter may be fainter in some embryos than others resulting in a miscount of cells. Also, more than one cell may be too close to each other and can be counted as one instead of two or three, resulting in fewer counts than expected. In addition, a GFP-expressing cell may appear as two cells and result in more counts than expected. Another question is why some of the wild-type embryos do not express the *cup-4*::GFP marker, even though it is a reporter that is integrated within a chromosome? Again, it may be that the marker is too faint to see, but it is also possible that the RNAi machinery within the animal silenced the new element within the chromosome, resulting in no expression in a small percentage of the embryos.

The next step was to determine if these cells are made by the MS blastomere. Feeding-induced RNAi was used to silence various genes responsible for the specification of MS tissues. As previously mentioned in the introduction, SKN-1 is a maternal transcription factor present at the top of the endomesoderm regulatory gene cascade and is essential in specification of the MS and E blastomeres. Previous studies show that in the absence of *skn-1*, embryos lack pharyngeal tissue 100% of the time and the MS cell adopts a C-like fate (Bowerman et al., 1992). Therefore, the prediction was that all coelomocytes will be missing in *skn-1* depleted embryos. Embryos expressing *cup-4*::GFP in a *skn-1(RNAi)* background produced 0.2 ± 0.05 coelomocytes (n = 124) compared to wild-type embryos (3.7 ± 0.2, n = 105, $P<0.001$). On the other hand, *med-1; med-2* (downstream target genes of SKN-1 in the EMS cell) double mutant embryos (strain MS887) did not express *cup-4*::GFP (0.02 ± 0.02; n = 43, $P<0.001$) (Fig. 11).

To further assess the expression pattern of the *cup-4*::GFP marker and confirm that coelomocytes are made by the MS cell, expression of *cup-4*::GFP was observed in mutant or heat-shock-induced embryos that produced mis-specified additional MS-like blastomeres. Over-expression of the *med-1* gene, using a heat-shock promoter fusion transgene (promoter that results in the gene to be expressed in all the cells of the embryo) (Stringham and Candido, 1993), resulted in ectopic expression of *cup-4*::GFP where embryos produced an average of 9.4 ± 0.4 coelomocytes (n = 60, $P<0.001$) (Fig. 11). To ensure that heat-shock itself was not causing any background expression, wild-type embryos expressing *cup-4*::GFP were also heat-shocked. These embryos produced the expected number of coelomocytes (4.0 ± 0.1, n = 74) and the number of coelomocytes made were not significantly different from those of wild-type embryos (3.7 ± 0.2, n = 105, P = 0.3). Ectopic production of MS tissues is observed when genes such as *pie-1* and *mex-1* are silenced, as well. Levels of SKN-1 protein are similar in the P_2 lineage as they are in EMS but its activity is repressed by another maternal factor, PIE-1. PIE-1 is essential for production of the germ cells, as well as, preventing P_2 and P_3 from responding to other factors involved in EMS and C specification (Mello et al., 1996). Another factor, MEX-1, acts to repress the activity of SKN-1 in the AB lineage, since *mex-1* mutant embryos develop ectopic MS tissues as a result of the AB lineage adopting an MS-like fate (Mello et al., 1992). Therefore, if the repressive roles of PIE-1 and MEX-1 are silenced and ectopic MS tissues will arise. Specifically, the C lineage adopts an MS-like fate in *pie-1* mutant embryos and the four AB grand-daughters adopt MS-like fates in *mex-1* mutant embryos. Expression of *cup-4*::GFP was observed in a

pie-1(RNAi) or *mex-1(RNAi)* background. In a *mex-1(RNAi)* background, the four AB grand-daughters cells adopt an MS-like fate, therefore *mex-1* mutant embryos should make about 20 coelomocytes (4 coelomocytes from MS and 16 from ABs). In addition, the P_3 blastomere of *pie-1(RNAi)* embryos adopts an MS-like fate and 8 coelomocytes are expected to be made (4 from MS and 4 from P_3). These predictions were slightly consistent in *pie-1(RNAi)* embryos which contained an average of 7.1 ± 0.2 coelomocytes ($n = 55$, $P = 10^{-22}$), however *mex-1* mutant embryos made an average of 11.2 ± 0.7 coelomocytes ($n = 38$, $P = 10^{-12}$) (Fig. 11). The difference in the number of coelomocytes counted in *pie-1(RNAi)* embryos (7.1 ± 0.2, n = 55) versus the number of coelomocytes expected (8) is not significant. But there is a large discrepancy between the number of coelomocytes counted in *mex-1(RNAi)* embryos (11.2 ± 0.7, n = 38) versus the number expected (20). One possible explanation is that in some embryos instead of all four AB grand-daughters producing coelomocytes, two AB grand-daughters may be making coelomocytes instead, resulting in an average of 12 coelomocytes. As a reminder, coelomocytes are derived from the MS granddaughters, MSpp and MSap. Since one pair of coelomocytes is made from a posterior MS granddaughter, this transformation may not have occurred in the AB lineage of *mex-1(RNAi)* embryos. Through the analysis of the embryonic lineage using a time-lapse, multi-focal plane recording system (a 4-D microscope) (Hird and White, 1993) it was found that lineage transformations occur more frequently in the anterior branches of the lineage and the posterior lineages are usually aberrant (Schnabel et al., 1996). Though, another possibility may be that a complete AB-to MS-like transformation does not occur

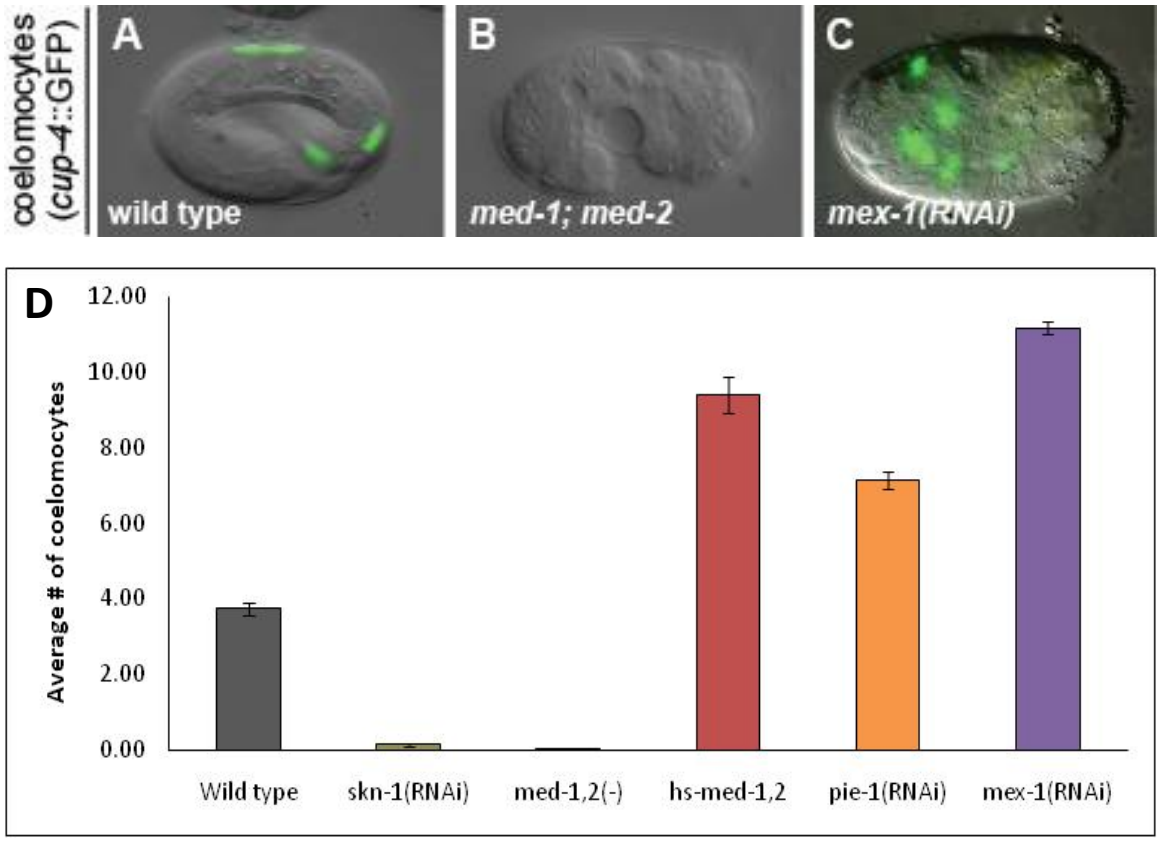


Fig. 11. The *cup-4*::GFP marker is expressed in the four embryonically-derived coelomocytes specified by the MS blastomere. (A, D) The *cup-4*::GFP marker (Patton et al., 2005) was found to be expressed in the four coelomocytes of a wild-type embryo (3.7 ± 0.2, n = 104). (B, D) In the absence of *med-1,2* 0.02 ± 0.02 coelomocytes were produced (n = 43) while s*kn-1* mutant embryos (not shown) produced 0.2 ± 0.05 coelomocytes (n = 124). (C, D) Embryos in which *med-1,2* is ectopically expressed, via heat-shock promoter, contain 9.4 ± 0.4 coelomocytes (n = 60). In addition, *pie-1(RNAi)* and *mex-1(RNAi)* embryos produce ectopic coelomocytes (7.1 ± 0.2, n = 55; 11.2 ± 0.7, n = 38, respectively). Figure panels A and B composited by M. Maduro from data acquired by myself.

resulting in fewer numbers of MS-like cells to be made in *mex-1* mutant embryos. In fact, similar findings were reported when body wall muscle cells were observed in *mex-1* mutant embryos (Schnabel et al., 1996). If all four AB grand-daughters adopted an MS-like fate, then embryos should express five times the MS fate. Therefore, if the MS cell in wild-type embryos produces 80 body wall muscle cells, then *mex-1* mutants are expected to produce five times the amount (120 body wall muscle cells). Surprisingly, in 1996 Schnabel et al. reported that the *mex-1* mutant embryos only produced an average of 40 body wall muscle cells, although the number of cells was highly variable ranging from 12 to 84 cells (Schnabel et al., 1996). It was concluded that mutant embryos randomly express MS-like patterns and the normal anterior/posterior polarity is usually not maintained in both the early cleavages and in the fates executed in the AB lineage (Schnabel et al., 1996). They reported that the observed lineage patterns in most of the mutant embryos do not correspond to a complete transformation of the AB descendants on the level of four AB cells and that the transformation may be manifested at any random level of the lineage (Schnabel et al., 1996). Therefore, the recovery of only a modest increase in ectopic coelomocytes in *mex-1(RNAi)* embryos is consistent with the incomplete ABx-to-MS transformations observed in *mex-1* mutant embryos.

In addition to these genetic knockdown experiments, laser ablation studies (technique described under materials and methods) were done to confirm that coelomocytes are MS-derived. This technique is done to answer the question of whether a particular cell (or cells) is solely responsible for specification of a subset of tissues (i.e., pharynx, muscle, etc.). If more than one blastomere produces that particular tissue, it is

best to ablate all the cells of the early embryo except for the blastomere being questioned, thus that cell is 'isolated' and continues to divide and differentiate. However, sometimes it is not required to ablate all the cells and isolate one. Instead, the cell in question can be the only one ablated. In this particular experiment the MS cell was isolated (all the cells were ablated except for MS) in wild-type embryos expressing *cup-4*::GFP and all embryos expressed four coelomocytes (n = 3) (Table 1). On the other hand, when the MS blastomere was ablated in wild-type embryos and remaining cells were able to divide, resulting in a partial embryo, *cup-4*::GFP expression was not observed (n = 3). Collectively, these results confirm that the *cup-4*::GFP marker behaves as expected for a coelomocyte marker in wild-type and mutant embryos under various conditions.

As previously mentioned, *tbx-35* is a downstream target of *med-1,2* and has been shown to be important for specification of at least some early MS-derived tissues (i.e., MS-derived pharynx and body wall muscle) (Broitman-Maduro et al., 2006). However, it is not known whether *tbx-35* is necessary for specification of later MS-derived tissues such as coelomocytes. We previously obtained a *tbx-35* chromosomal mutation (*tm1789*) from the laboratory of Shohei Mitani (Tokyo, Japan). The *tm1789* strain has been genetically manipulated in our laboratory, such that it carries a homozygous *tbx-35(tm1789)* genotype balanced by an extrachromosomal array consisting of *tbx-35(+)* and an *unc-119*::CFP reporter. The *unc-119*::CFP reporter allows for identification of embryos that specifically lack the balancer array, which is lost at a predictable rate for a given array (~30-70%). The *unc-119* gene is expressed from early embryogenesis (~100 cell-stage) through adulthood and is used to mark embryos whose

Table 1. MS-dependent tissues produced in wild-type and mutant embryos

Genotype	Pharynx Cells[1] (*pha-4*::GFP)	Pharynx Muscles[2] (*ceh-22*::GFP)	Muscle Cells (*hlh-1*::GFP)	Coelomocytes (*cup-4*::GFP)
wild type	50.0 ± 0.9 (21)	12.8 ± 0.1 (37)	44.7 ± 1.1 (20)	3.7 ± 0.2 (105)
skn-1(RNAi)	4.8 ± 0.4 (20)	0.0 ± 0.0 (165)	nd	0.15 ± 0.04 (124)
pal-1(RNAi)	49.5 ± 0.8 (10)	11.7 ± 0.3 (12)	21.6 ± 0.9 (13)	3.7 ± 0.1 (103)
pop-1(RNAi)	nd	nd	nd	0.0 ± 0.0 (50)
glp-1(RNAi)	23.1 ± 0.6 (15)	5.7 ± 0.2 (38)	nd	nd
tbx-35(tm1789) 15°C	40.6 ± 1.2 (17)	5.9 ± 0.3 (24)	37.3 ± 1.6 (10)	3.8 ± 0.2 (28)
tbx-35(tm1789) 23°C	35.7 ± 0.8 (16)**	5.2 ± 0.2 (46)*	34.8 ± 2.4 (10)	3.3 ± 0.4 (20)
tbx-35(tm1789); glp-1(RNAi) 15°C	6.6 ± 0.5 (23)	2.0 ± 0.4 (26)	nd	nd
tbx-35(tm1789); glp-1(RNAi) 23°C	1.1 ± 0.3 (32)**	1.0 ± 0.2 (39)*	nd	nd
tbx-35(tm1789); pal-1(RNAi) 15°C	38.8 ± 0.7 (15)	5.1 ± 0.3 (14)	8.4 ± 1.0 (17)	2.2 ± 0.2 (47)
tbx-35(tm1789); pal-1(RNAi) 23°C	35.6 ± 1.0 (14)*	4.7 ± 0.3 (17)	5.7 ± 0.5 (40)*	0.6 ± 0.1 (49)**
ceh-51(tm2123)	47.8 ± 0.9 (17)	9.2 ± 0.2 (10)	42.4 ± 1.4 (10)	2.1 ± 0.1 (53)
ceh-51(tm2123); pal-1(RNAi)	nd	nd	19.3 ± 0.5 (11)	2.5 ± 0.1 (84)
med-1(ok804); med-2(cx9744)	31.3 ± 0.6 (26)	4.1 ± 0.2 (32)	31.0 ± 2.7 (10)	0.07 ± 0.03 (34)
ceh-51(tm2123); tbx-35(tm1789)	30.2 ± 0.5 (44)	4.4 ± 0.2 (18)	30.1 ± 1.0 (14)	0.19 ± 0.04 (124)*
med-1(ok804); med-2(cx9744); glp-1(RNAi)	1.4 ± 0.4 (14)	0.3 ± 0.1 (31)	nd	nd
ceh-51(tm2123); tbx-35(tm1789); glp-1(RNAi)	1.9 ± 0.5 (26)	0.5 ± 0.1 (52)	nd	nd
med-1(ok804); med-2(cx9744); pal-1(RNAi)	nd	nd	3.8 ± 0.5 (13)	nd
ceh-51(tm2123); tbx-35(tm1789); pal-1(RNAi)	nd	nd	3.9 ± 0.4 (15)	nd

Strains were grown at 20-23°C unless otherwise indicated. Data are shown as mean ± SEM. Asterisks denote $0.01 < p < 0.05$ (*) or $p < 0.01$ (**) in a *t*-test with the experiment immediately above.

[1]Only pharynx expression of *pha-4*::GFP, anterior to the gut (when present), was scored.

[2]The anatomy of the pharynx was considered in assigning expression to particular muscle cells.

Table composited by M. Maduro from data acquired by myself, M. Maduro, and G. Broitman-Maduro (Broitman-Maduro et al., 2009)

overall genotype is *tbx-35(+)*. This strain was crossed with another that contained the *cup-4*::GFP marker to construct the *tbx-35(tm1789); cup-4*::GFP strain (details explained in Materials and Methods).

To assess whether *tbx-35* is necessary to make coelomocytes, embryos with CFP expression (indicating that *tbx-35* is rescued) and no CFP (indicating the absence of *tbx-35*) were scored for number of coelomocytes. Surprisingly, expression of *cup-4*::GFP continued to be present in *tbx-35* mutant embryos grown at 20°C with an average of 3.3 ± 0.4 (n = 20) coelomocytes (Fig.12B, D). Interestingly, the number of coelomocytes produced in *tbx-35* mutant embryos was not significantly different from the number of coelomocytes produced in wild-type embryos (3.7 ± 0.2, n = 105, P = 0.3) (Fig. 12A, D). Over-expression of *tbx-35* resulted in ectopic expression of *cup-4*::GFP with embryos producing an average of 14.1 ± 1.2 (n = 43, P<0.001) coelomocytes (Fig. 12C, D). As previously noted, *skn-1(RNAi)* embryos did not make coelomocytes (0.2 ± 0.05, n = 124), however the number of coelomocytes produced in hs-*tbx-35* embryos in a *skn-1(RNAi)* background increased to 2.2 ± 0.5 coelomocytes (n = 95, $P<10^{-8}$) (Fig. 12E). These results suggest that *tbx-35* is sufficient to specify for coelomocytes even in the absence of *skn-1*. Expression of *cup-4*::GFP was also analyzed in *tbx-35* mutant embryos with *mex-1(RNAi)* and *pie-1(RNAi)* backgrounds to determine if ectopic expression of coelomocytes in *mex-1* and *pie-1* RNAi embryos depends on *tbx-35*. Expression of *cup-4*::GFP was reduced dramatically under both conditions, in which *tbx-35(-)*; *mex-1(RNAi)* embryos produced an average of 1.1 ± 0.2 coelomocytes (n = 22) compared to *mex-1*(RNAi) embryos which made an average of 11.2 ± 0.7 (n = 38, P<0.001). In addition,

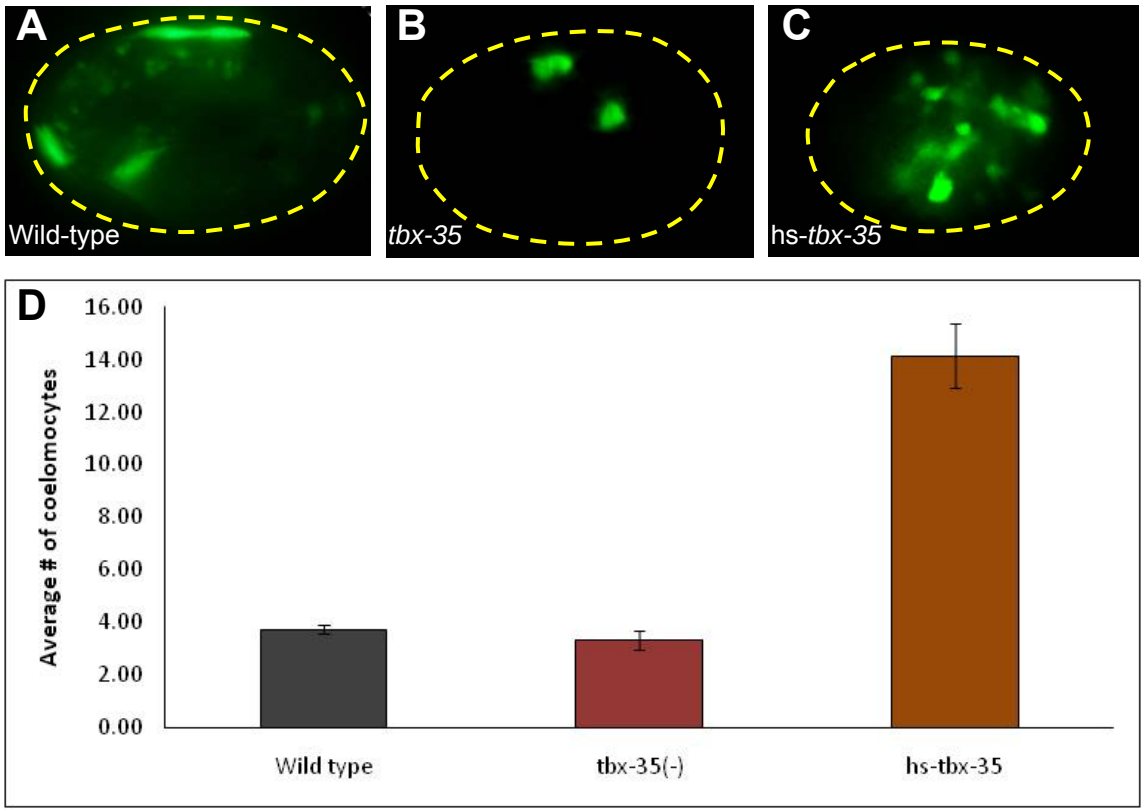
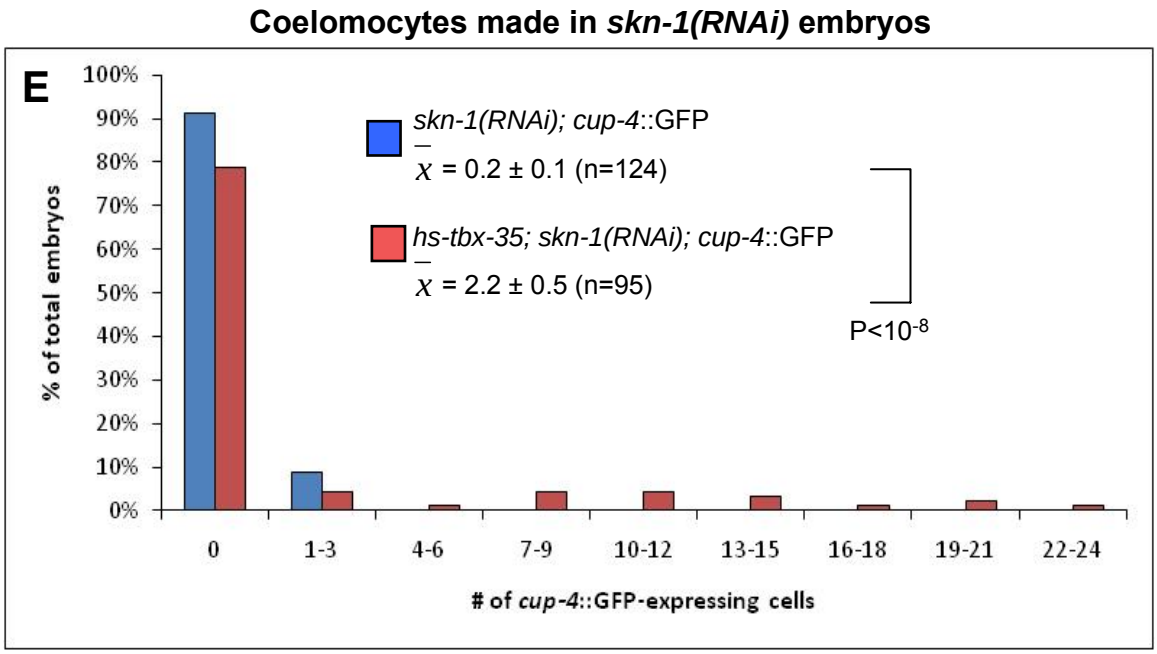


Fig. 12. TBX-35 is sufficient but not necessary for coelomocyte specification. (A, B, D) *tbx-35* mutant embryos continued to produce coelomocytes (3.3 ± 0.4, n = 20) and was not significantly different from wild-type embryos (3.7 ± 0.2, n = 105, P = 0.3). (C, D) However, over-expression of *tbx-35* resulted in ectopic expression of *cup-4*::GFP marker (14.1 ± 1.2, n = 43), while hs-*tbx-35* embryos also continue to make coelomocytes in a *skn-1(RNAi)* background (2.2 ± 0.5, n =95), unlike *skn-1(RNAi*) embryos which made almost no coelomocytes (0.2 ± 0.1, n = 124) (E).

Fig. 12 continued



tbx-35(-); *pie-1(RNAi)* embryos contained an average of 2.5 ± 0.3 coelomocytes (n = 22) compared to *pie-1(RNAi)* embryos which produced 7.1 ± 0.2 (n = 55, P<0.001). Although expression of *cup-4*::GFP is dramatically reduced, there is still expression of *cup-4*::GFP in the double mutant embryos. Therefore, ectopic expression of *cup-4*::GFP in *mex-1(RNAi)* and *pie-1(RNAi)* backgrounds is not completely dependent on *tbx-35*, suggesting that there is at least one other factor responsible for coelomocyte specification. These results indicate that *tbx-35* is sufficient but not necessary for specification of coelomocytes and acts upstream of coelomocyte specification, however at least one other factor, besides *tbx-35*, is involved in specification of these MS-derived tissues.

Identification of other factors responsible for specification of coelomocytes

There are nearly 21 related T-box genes identified in the *C. elegans* genome (Chervitz et al., 1998; Ruvkun and Hobert, 1998). Previous studies have shown several T-box genes to have overlapping functions. For example, *tbx-8* and *tbx-9*, together, control several aspects of embryonic morphogenesis and patterning (Pocock et al., 2004). Also, *tbx-37* and *tbx-38* are both required for proper cell fate specification in the ABa lineage and development of the anterior pharynx (Good et al., 2004). In addition, other regulatory genes have been found to be redundant [i.e., *med-1* and *med-2*; *end-1* and *end-3* (Maduro et al., 2001; Zhu et al., 1997)]. Therefore, I was interested to determine if the other parallel factor that may work with *tbx-35* is also a T-box gene.

Approximately thirteen T-box genes were reported to have unknown functions (WormBase WS210). Since *tbx-35* is a direct target of MED-1,2, we continued to identify candidate MED-1,2 targets by looking for MED binding sites, 5'-RAGTATAC-3' (Broitman-Maduro et al., 2005). From the thirteen T-box genes, *tbx-33* and *tbx-41* were found to have several MED binding sites near the promoter. Therefore, it was of interest to determine if these genes are expressed in the MS blastomere and if they contribute to specification of MS-derived tissue. In-situ hybridization was done to observe the mRNA expression, and we found *tbx-33* expressed in majority of the blastomeres of the early embryo except for E and P_2 (Fig. 13A). Expression of *tbx-41*, however, was not detected in the embryo (Fig. 13B). Although *tbx-33* is expressed outside the MS lineage, as well as within it, it was still of interest to determine if the absence of *tbx-33* resulted in a phenotype. The role of *tbx-33* in specification of coelomocytes was examined and the first approach was to silence the expression of *tbx-33* via feeding-induced RNAi (cloning explained under Materials and Methods). Using the MS862 strain (*tbx-35*; *unc-119*::CFP; *cup-4*::GFP), *tbx-33(RNAi)* was induced by feeding and coelomocytes were scored in the double mutant embryos. These embryos produced an average of 1.7 ± 0.2 coelomocytes (n = 39), which is about a 2-fold decrease compared to *tbx-35* mutant embryos (3.3 ± 0.4, n = 20, $P<10^{-8}$). Further analysis, including control experiments with *tbx-33(RNAi)* alone were not performed and was not observed in other lineages however these preliminary results do suggest a possible role for *tbx-33* in specification of coelomocytes. Typically when genes are functionally redundant the absence of one gene may result in a normal phenotype, since the presence

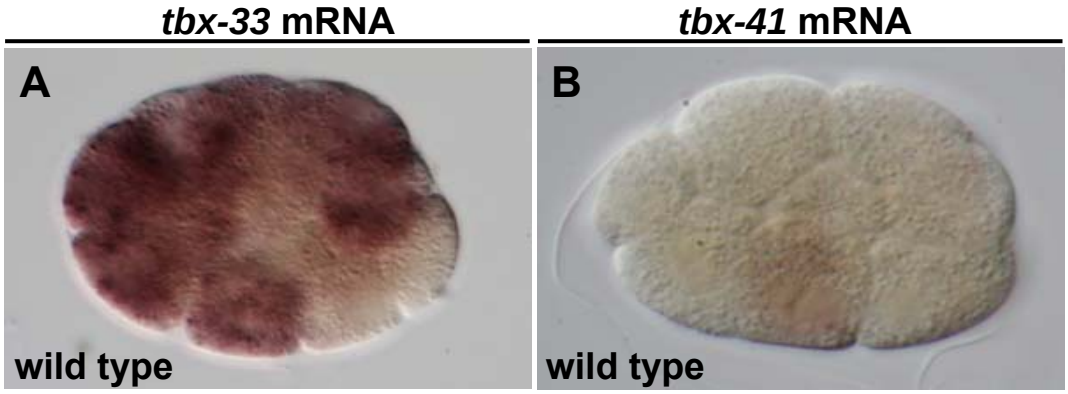


Fig. 13. Endogenous expression of *tbx-33* and *tbx-41*. (A, B) In situ hybridization showing endogenous expression of *tbx-33* and *tbx-41*, which were proposed to possibly be redundant with *tbx-35*, since both, *tbx-33* and *tbx-41*, contained several *med-1,2* binding sites. Endogenous expression of *tbx-33* was observed in all cells except for the E descendants and the P_2 lineage. On the other hand, *tbx-41* mRNA was not found to be expressed in the early embryo. In situ hyrbidization and images done by G. Broitman-Maduro.

of the other gene is able to compensate for the missing gene. But *tbx-35* mutants do not survive and do not escape lethality; they arrest during embryonic development. Therefore, it is likely that the other parallel factor is not a redundant factor that can compensate for the loss of TBX-35 function. Although the preliminary results suggest that *tbx-33(RNAi)* enhances the *tbx-35(tm1789)* mutant phenotype, it may simply be that less coelomocytes are made because *tbx-33(RNAi)* embryos, alone, arrest as early as the ~200 cell-stage, which is not far enough for all the cells to differentiate or show complete morphogenesis. Therefore, it was still of interest to investigate other candidate factors that may work in parallel with *tbx-35*.

One of the maternal genes, *pal-1*, has been shown to be a blastomere-specification gene required for normal development of the somatic descendants of P_2 (Hunter and Kenyon, 1996). Through two mechanisms *pal-1* activity is restricted to the P_2 lineage. First, translation of *pal-1* is inhibited in the anterior region of the embryo by an RNA-binding protein, MEX-3, which binds to the 3'-UTR of *pal-1*. This results in *pal-1* to be expressed in P_2 and EMS blastomeres. However, as a second mechanism SKN-1, which functions in EMS, is proposed to block *pal-1* activity resulting in it to act only in P_2 (Hunter and Kenyon, 1996). But despite this, PAL-1 has been found to have a cryptic function in the descendant of EMS, the E blastomere. For example, in embryos that fail to make endoderm as a result of aberrant activity in the SKN-1 pathway, the E cell adopts a C-like fate and ectopic C-derived body wall muscle and hypodermis are produced (Bowerman et al., 1992; Maduro et al., 2001). The E- to C-like transformation that results from the presence of PAL-1, suggests that PAL-1 does function in the E cell in the

absence of SKN-1 (Bowerman et al., 1992; Hunter and Kenyon, 1996; Maduro et al., 2001). A previous study has also demonstrated that PAL-1 does in fact have a role in endoderm specification (Maduro et al., 2005b). In the absence of both SKN-1 and POP-1, some embryos continue to make gut, however when PAL-1 is also absent (in addition to SKN-1 and POP-1) none of the embryos make gut (Maduro et al., 2005b). Collectively, it is clearly demonstrated by such findings, that PAL-1 has a cryptic function in specifying gut in the E cell. This led to question the possibility of PAL-1 having a function in the MS blastomere.

Since coelomocytes continue to be made in *tbx-35* mutant embryos, I was interested to determine whether *pal-1* may contribute to coelomocyte specification in the absence of *tbx-35*. In the absence of *pal-1*, alone, embryos continue to make 4.31 ± 0.12 coelomocytes (n = 64). However, *tbx-35(-); pal-1* RNAi embryos produced an average of 0.6 ± 0.1 coelomocytes (n = 49), compared to *tbx-35* mutant embryos which made 3.3 ± 0.4 (n = 20, $P<0.001$). The results suggest that PAL-1 and TBX-35 may provide overlapping function in specification of coelomocyte precursors. To determine if *pal-1* is sufficient for coelomocyte specification, wild-type and *tbx-35* mutants were fed bacteria expressing *mex-3* dsRNA. As described earlier, MEX-3 is an RNA-binding protein that prevents the protein expression of *pal-1* in the AB blastomeres. By silencing the function of MEX-3, ectopic PAL-1 protein expression will result, allowing for examination of the sufficiency of PAL-1 to ectopically specify for coelomocytes. In the absence of *mex-3*, wild-type embryos expressing the *cup-4*::GFP marker made an average of 3.6 ± 0.1 coelomocytes (n = 127). However, *tbx-35(-)*; *mex-3* RNAi embryos expressed an average

of 0.1 ± 0.1 coelomocytes (n = 49), suggesting that *pal-1* is not sufficient to specify for coelomocytes and that MEX-3, perhaps, contributes to coelomocyte specification in MS. However, it is likely the possibility that the decrease in the number of coelomocytes may be due to an increase of PAL-1 expression in MS, making the *tbx-35(-)* MS-to-C transformation more expressive. Collectively, the results demonstrate that *pal-1* is neither necessary nor sufficient for coelomocyte specification but may have a cryptic role in the MS blastomere in the absence of *tbx-35*. The fact that ectopic PAL-1 expression via *mex-3(RNAi)* in the *tbx-35* mutant background resulted in embryos making almost no coelomocytes compared to *tbx-35*; *pal-1(RNAi)* double mutants, remains difficult to explain with current models. However, these findings are corroborated by results of laser ablation studies done in *tbx-35* mutant embryos rescued by the *tbx-35* transgene array expressing *cup-4*::GFP. In embryos in which ABa was ablated an average of 2.6 ± 0.54 (n = 5) coelomocytes were expressed. Together, the results suggest the possibility that ABa may have a role in signaling to MS, since ablation of the ABa blastomere affects the production of coelomocytes, or the possibility that the fate of MS changes in a *mex-3(RNAi)* background. However, further experiments are required to determine if this possibility holds true.

The tbx-35(tm1789) mutants resemble a more wild-type phenotype when grown at lower temperatures

Since most of the DNA-binding domain is deleted in the *tbx-35(tm1789)* mutant, these embryos were found to exhibit a temperature-sensitive phenotype. In other words,

the mutant phenotype of embryos carrying a temperature-sensitive mutation is apparent at a non-permissive temperature (in this case at a higher temperature range). However, the mutant phenotype is not as apparent when grown at a permissive temperature (a lower temperature range). The initial observation was that *tbx-35(tm1789)* embryos had a mutant phenotype, since they were grown and maintained at 20-25ºC. However, these animals were also grown at 15ºC and it was found that the majority of the embryos elongated, hatched, and developed a normal pharynx (features that were not found in most mutant animals raised at 20-25ºC). Furthermore, they arrest at the L1 stage unlike animals which arrest earlier when raised at higher temperatures. Therefore, it was of interest to determine if the mutant embryos also produce the normal number of coelomocytes when grown at lower temperatures.

The *tbx-35(tm1789)* embryos, carrying the *cup-4*::GFP reporter, were grown at 15°C and it was found that these mutant embryos expressed as many coelomocytes (3.8 ± 0.2, n = 28) as wild-type embryos (3.7 ± 0.2, n = 105, P = 0.3), which is consistent with previous findings explained above. It was described earlier that *tbx-35(tm1789); pie-1(RNAi)* embryos, raised at 20ºC produced 2.5 ± 0.3 (n = 22) coelomocytes compared to *pie-1(RNAi)* embryos (7.1 ± 0.2, n = 55, P<0.001). However, expression of *cup-4*::GFP increased in *tbx-35(-)*; *pie-1(RNAi)* embryos raised at 15ºC and these embryos contained an average of 5.9 ± 0.2 coelomocytes (n = 54), compared to *tbx-35(-); pie-1(RNAi)* embryos raised at 20ºC (2.5 ± 0.3, n = 22, P<0.001). In addition, *tbx-35; pal-1(RNAi)* embryos grown at 15°C were able to produce more coelomocytes (2.2 ± 0.2, n = 47) and reach a phenotype closer to wild-type, unlike *tbx-35; pal-1(RNAi)* embryos grown at

20ºC (0.61 ± 0.14, n = 49, P<0.001). These findings provide further evidence that *tbx-35* mutant embryos raised at lower temperatures develop more similarly to wild-type embryos.

Role of NK-2 class homeobox gene, ceh-51, in MS specification

Recently, we have reported that the NK-2 class homeobox gene *ceh-51* is a direct target of TBX-35 and at least one other factor (Broitman-Maduro et al., 2009). The gene Y80D3A.3 was identified as a candidate early MS lineage gene from embryonic transcriptome analysis (Baugh et al., 2003). In situ hybridization studies were conducted to determine the developmental time point in the early embryo *ceh-51* is gene expressed. As expected, transcript levels were abundant in the early MS lineage (MS^2 to MS^4 stage). A *ceh-51*::GFP reporter was also constructed to observe its expression pattern at the protein level. Thus, *ceh-51*::GFP was observed at the MS^4- and MS^8-cell stage (Fig. 14A, B, E, F) (Broitman-Maduro et al., 2009). Collectively, *ceh-51* was expressed at the right time and place in the early embryo.

To confirm the hypothesis that the expression of *ceh-51* is dependent on the SKN-1/MED-1,2 pathway, the *ceh-51*::GFP reporter was observed in these mutant backgrounds. Thus, in the absence of *skn-1* and *med-1,2*, embryos did not express *ceh-51*::GFP (Fig. 14 G), as expected (Broitman-Maduro et al., 2009). However, ectopic *ceh-51*::GFP was observed in *mex-1* and *pie-1* RNAi backgrounds (Fig. 14I, J), since AB and C lineages transform to an MS-like fate, respectively (Broitman-Maduro et al., 2009).

Together, these results demonstrate that *ceh-51* is regulated through the SKN-1 specification pathway.

As previously described, the EMS daughter cells, MS and E, divide asymmetrically as a result of a Wnt/MAPK signal originating from P_2 to EMS. If this signal is absent then the EMS cell will give rise to two MS-like daughter cells. This suggests that the Wnt/MAPK signal, notably the TCF/POP-1 transcription factor, may be repressing MS-specific factors in the E cell in order for the E fate to be specified. Thus, it was of interest to find out if the MS-specific genes, *tbx-35* and *ceh-51*, express in the E lineage in the absence of *pop-1*. Initially, the expression pattern of *tbx-35* was observed in *pop-1(RNAi)* embryos using a *tbx-35*::GFP reporter and it was found that *tbx-35*::GFP expression persisted in the MS lineage (Broitman-Maduro et al., 2006). However through personal communication with the Rueyling Lin lab, a *tbx-35*::GFP reporter that contained more of the *tbx-35* promoter region was used and found to be expressed in the E lineage in the absence of *pop-1* [this result was also reproduced by others in our lab (Owraghi et al., 2009)]. Recently, the expression of *ceh-51*::GFP was also observed in *pop-1(RNAi)* embryos. Since *tbx-35*::GFP expressed in both MS and E it was of no surprise to also find *ceh-51*::GFP expressed in both MS and E lineages in *pop-1(RNAi)* embryos (Fig. 14D, H) (Broitman-Maduro et al., 2009). Collectively, these findings show that even though an MS-to-E transformation occurs in *pop-1* mutant embryos MS-specific factors continue to express in both the MS and E lineages, but fail to specify MS-specific tissues and instead *pop-1* mutants produce ectopic gut due to the presence of *end-*

1 and *end-3* (further experiments and analysis are followed and described in the following chapter).

To determine if *ceh-51* is a direct target of *tbx-35*, expression of *ceh-51* was observed in a *tbx-35* mutant background, as well as, embryos over-expressing *tbx-35*. Over-expression of *tbx-35* resulted in ectopic expression of *ceh-51* (Fig. 14C) (Broitman-Maduro et al., 2009) however, *ceh-51* still expressed in half of the embryos in *tbx-35* mutants (Fig. 14K) (Broitman-Maduro et al., 2009). Therefore, the results show that *tbx-35* is sufficient but not necessary for activation of *ceh-51*. It is also likely that an additional MS-specific factor upstream of *ceh-51* may act in parallel with *tbx-35* to activate *ceh-51*. Since the *ceh-51*::GFP reporter expressed in both MS and E lineage in the absence of *pop-1* and also continued to be expressed in the MS lineage in *tbx-35* mutants, we wanted to determine if *ceh-51*::GFP expression would diminish if both *tbx-35* and *pop-1* were absent. The expression of *ceh-51*::GFP was not detected in *tbx-35(-); pop-1(RNAi)* embryos (Fig. 14L), suggesting that activation of *ceh-51* in a *tbx-35* mutant background depends on POP-1 (Broitman-Maduro et al., 2009).

As described earlier, pharynx and body muscle are not only made by the MS cell, but the anterior pharynx, is made by the AB lineage through the activity of GLP-1, while body muscle is made by the C and D lineage through the activity of the maternal factor PAL-1. In addition, SKN-1 is required for specification of pharynx, MS-derived body wall muscle cells and E-derived gut. Therefore, to determine if a particular gene is sufficient to make these tissue types it is best to overexpress the gene so that the

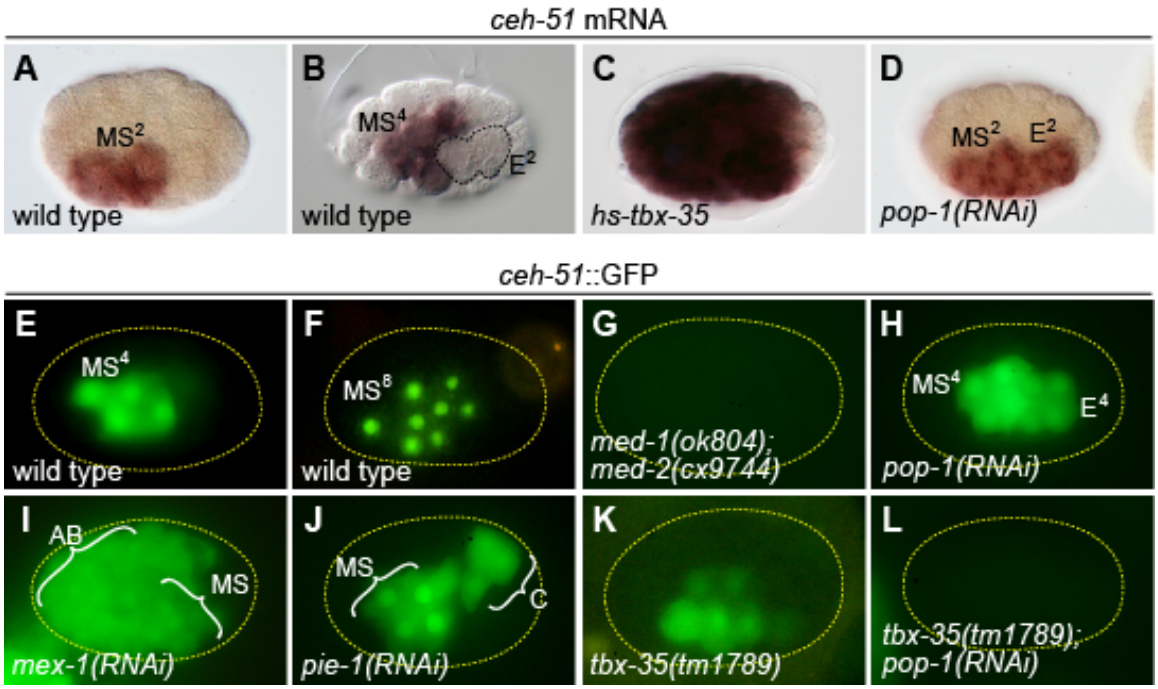


Fig. 14. Expression of *ceh-51*. (A, B) In situ hybridization (performed by G. Broitman-Maduro) shows *ceh-51* transcripts to be found in the MS daughter cells (MS^2) (A), and in the MS granddaughters (MS^4) (B). (C) Following heat shock of hs-*tbx-35* embryos, *ceh-51* was found to be ectopically expressed. (D) Eighty-six percent (n = 44) of *pop-1(RNAi)* embryos expressed *ceh-51* mRNA in both MS and E daughters. (E) Embryos transgenic for a *ceh-51*::GFP transcriptional reporter were found to be expressed at MS^4 and persists in later MS descendants. (F) A translational *ceh-51*::GFP::CEH-51 fusion shows strong nuclear accumulation at MS^8. (G) *ceh-51*::GFP was not detected in *med-1(ok804); med-2(cx9744)* embryos (n = 84). (H) Sixty-six percent (n = 41) of *pop-1(RNAi)* embryos showed *ceh-51*::GFP in both MS and E lineages, while the remaining *pop-1(RNAi)* embryos were similar to wild type. (I, J) *mex-1(RNAi)* (I) and *pie-1(RNAi)* (J) embryos displayed ectopic *ceh-51*::GFP in AB and C descendants. (K) In *tbx-35(tm1789)* embryos, *ceh-51*::GFP expression was undetectable (52%, n = 89) or delayed until past the MS^8) stage (48%) and at lower levels. (L) *ceh-51*::GFP was not detected in *tbx-35(tm1789); pop-1(RNAi)* embryos (n = 49). Figure composited by M. Maduro from data acquired by myself, M. Maduro and G. Broitman-Maduro. Legend modified from Broitman-Maduro et al., 2009.

transcripts will be present throughout the entire embryo. In addition, a functional domain of the gene can be deleted to determine if it is required to make the specific tissue-type. This technique will show that without its regulators, the gene product is still able to give rise to specific tissues. For example, to determine if *ceh-51* is sufficient for specification of MS-derived tissues, strains expressing the heat-shock *ceh-51* transgene and gene reporters such as those for pharynx muscle, *ceh-22* (Okkema and Fire, 1994); pharynx myosin *myo-2* (Okkema et al., 1993); and body muscle, *unc-120* and *myo-3* were constructed and used in a *skn-1(RNAi)*, *skn-1(RNAi); pal-1(RNAi)*, or *skn-1(zu67); pal-1(RNAi)* background. Over-expression of *ceh-51* did not result in significant expression of pharynx markers (0.7 ± 0.2, n = 93) compared to *skn-1(RNAi)* embryos alone in which no transgene was present (0.0 ± 0.0, n = 106) (Fig. 15A, B, F, G, K), suggesting that CEH-51 is not sufficient to specify pharynx in the absence of *skn-1* (Broitman-Maduro et al., 2009). On the contrary, in *skn-1(zu67); pal-1(RNAi)* embryos, in which all body muscle specification is blocked, ectopic expression of *myo-3* mRNA (Miller et al., 1986) was detected when *ceh-51* was over-expressed (Fig. 15D, I) (Broitman-Maduro et al., 2009). In addition, hs-*ceh-51* embryos expressed ectopic *unc-120*::GFP [also a muscle gene reporter (Fukushige et al., 2006)] in *skn-1(RNAi); pal-1(RNAi)* embryos (16.8 ± 2.3, n = 46), while *skn-1(RNAi); pal-1(RNAi)* embryos in which *ceh-51* was not over-expressed produced an average of 2.8 ± 0.4 (n = 66, $P<10^{-6}$) muscle cells (Fig. 15C, H, L) (Broitman-Maduro et al., 2009). Therefore, *ceh-51* is sufficient to specify for muscle in the absence of *skn-1*. Likewise, *ceh-51* was found to be sufficient to specify for coelomocytes in a *skn-1(RNAi);* hs-*ceh-51* embryos (2.1 ± 0.3, n = 166), producing a

range of 0-15 coelomocytes, whereas *skn-1(RNAi)* embryos without the hs-*ceh-51* transgene did not express coelomocytes (0.05 ± 0.03, n = 111, $P<10^{-8}$) (Fig. 15E, J, M) (Broitman-Maduro et al., 2009). These findings were similar to what was found in hs-*tbx-35*; *skn-1(RNAi)* embryos (2.2 ± 0.5, n = 95) producing a range of 0-22 coelomocytes (Fig 12E). The hs-*tbx-35; skn-1(RNAi)* embryos also produced ectopic muscle (13.2 ± 1.7, n = 87) and ectopic pharynx (4.4 ± 0.8, n = 141) (Broitman-Maduro et al., 2006), unlike hs-*ceh-51; skn-1(RNAi)* embryos which didn't produce many pharynx cells (0.7 ± 0.2, n = 93).

Since *ceh-51* is sufficient to make muscle and coelomocytes the next step was to determine if *ceh-51* is necessary for specification of these MS-specific tissues. To assess the requirement of *ceh-51*, embryos with *ceh-51(RNAi)* and *ceh-51(tm2123)* (gift from Shohei Mitani, National Bioresource project, Japan) mutants were examined. Observing the phenotype of *ceh-51(RNAi)* embryos and *ceh-51(tm2123)* embryos allows for a comparison of which method results in a more penetrant, lethal phenotype. Thus, the first set of observations involved gonadal injection of *ceh-51* dsRNA, in which 47% (n = 70) of progeny arrested as uncoordinated L1 larvae, whereas the remainder appeared normal (50%) or arrested as early embryos (3%) (Broitman-Maduro et al., 2009). However, the putative null mutant, *tm2123*, resulted in a fully penetrant recessive zygotic L1 arrest (Broitman-Maduro et al., 2009). Therefore, the *ceh-51(tm2123)* mutants were used for the subsequent experiments. In *ceh-51(tm2123)* mutant embryos, pharynx tissue is specified but does not develop properly. The pharynx muscle marker, *ceh-22*::GFP was used and was detected both inside and outside the pharynx, suggesting defective

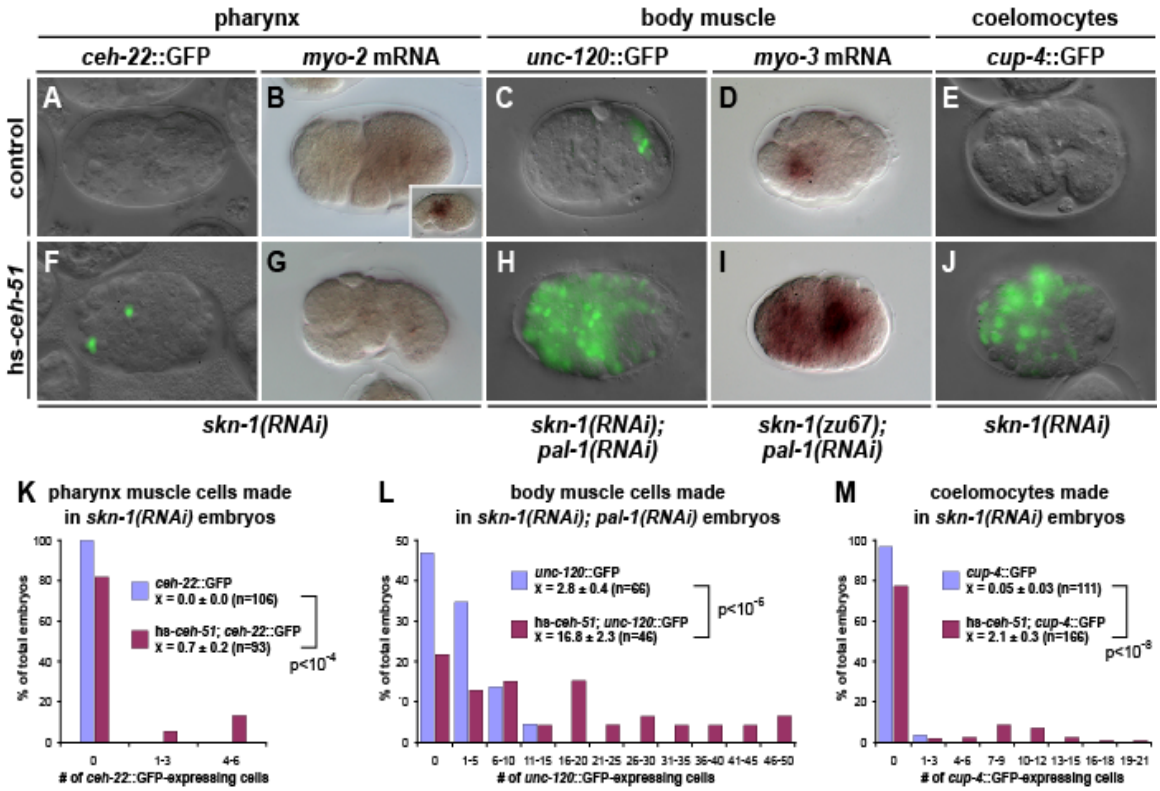


Fig. 15. Over-expression of CEH-51 results in specification of MS-derived cell types. (A, F) A small number of cells expressing *ceh-22*::GFP are observed in hs-*ceh-51; skn-1(RNAi*) embryos, while no expression is observed in *skn-1(RNAi*) embryos. (B, G) Expression of the pharynx muscle gene *myo-2* is absent in both *skn-1(RNAi)* and hs-*ceh-51; skn-1(RNAi)* embryos. The inset in B shows wild-type expression of *myo-2*. (C, H) Many hs-*ceh-51* embryos display *unc-120*::GFP-expressing cells in a *skn-1(RNAi); pal-1(RNAi)* background, which depletes embryos of MS- and C-derived body wall muscle cells (Hunter and Kenyon, 1996). (D, I) In *skn-1(zu67); pal-1(RNAi)* embryos, heat shock of *ceh-51* results in the generation of many cells expressing the muscle myosin gene, *myo-3*. One hundred percent (n = 79) of non-transgenic, heat shocked embryos resembled those shown in D, whereas 53% (n = 53) of heat shocked transgenics ectopically expressed the *myo-3* transcript (I). (E, J) hs-*ceh-51* embryos express ectopic coelomocytes in the *skn-1(RNAi)* background. (K-M) Bar charts summarizing the hs-*ceh-51* data. In situ hybridization done by G. Broitman-Maduro. Figure composited by M. Maduro from data acquired by myself, M. Maduro and G. Broitman-Maduro. Legend modified from Broitman-Maduro et al., 2009.

pharynx structure (Fig. 16D, E) (Broitman-Maduro et al., 2009). In addition, the pharynx was found to be detached in certain regions in majority of embryos. To rule out the effects of GFP reporters, the actin filaments of pharynx tissue was observed using an Alexa-488 labeled antibody against phalloidin (Molecular Probes), which directly binds to actin filaments (Franks et al., 2006). In the *ceh-51(tm2123)* mutant embryos the actin filaments of the pharynx musculature appeared to be disorganized compared to wild-type animals (Fig. 16C, F**)**. Despite the abnormal development of pharynx tissue, the number of pharynx cells produced in *ceh-51* mutants was found to be similar to wild-type animals (Table 1) (Broitman-Maduro et al., 2009).

To observe the requirement of *ceh-51* for specification of body muscle cells the *hlh-1*::GFP reporter was used (Krause et al., 1994). The number of body muscle cells was found to be slightly reduced in *ceh-51(tm2123)* mutant embryos (42.4 ± 1.4, n = 10) compared to wild-type (44.7 ± 1.1, n = 20) (Table 1) (Broitman-Maduro et al., 2009). In addition, C-derived body muscle was depleted using *pal-1(RNAi)* in the *ceh-51(tm2123)* mutant background. Since there was not a significant decrease in the number of body muscle cells in *ceh-51(tm2123)* mutants alone, a significant decrease in the number of body muscle cells is not expected in *ceh-51(tm2123); pal-1(RNAi)* embryos either. The *ceh-51(tm2123); pal-1(RNAi)* embryos contained 19.3 ± 0.5 (n = 11) *hlh-1*::GFP expressing cells while *pal-1(RNAi)* embryos, alone, produced 21.6 ± 0.9 (n = 13, P=0.2) cells (Broitman-Maduro et al., 2009). These results also suggest that *ceh-51*, alone, is not necessary for specification of the MS-derived body muscle fate, unlike *tbx-35* (Broitman-Maduro et al., 2006).

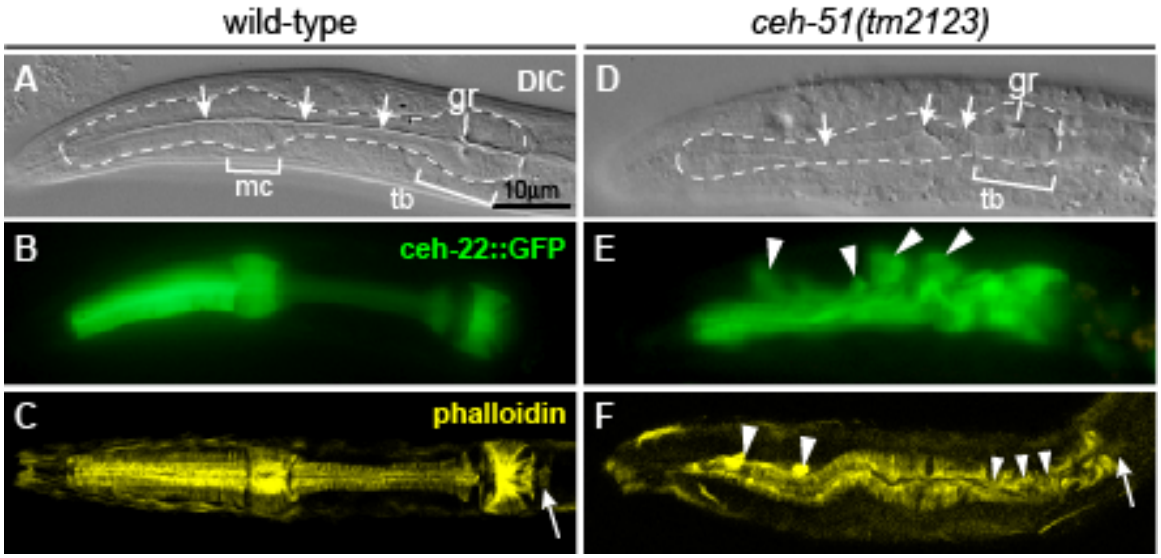


Fig. 16. *ceh-51* mutants arrest as larvae with pharynx structural defects. Pharynxes were visualized by DIC optics (A, D), *ceh-22*::GFP expression (B, E) (Okkema and Fire, 1994) or phalloidin staining (C, F) (Franks et al., 2006). In the DIC panels, the lumen (arrows), grinder (gr), metacorpus (mc) and terminal bulb (tb) are indicated and the pharynx is outlined (dashed line). (A-C) Wild-type pharynx. (D-F) *ceh-51(tm2123)* pharynxes show lumen abnormalities and an indistinct metacorupus (D). Protursions accumulate GFP outside the pharynx, suggesting a defect in pharynx integrity (E). Phalloidin staining shows actin filament accumulations (large arrowheads), lumen abnormalities (small arrowheads) and an abnormal terminal bulb (arrow). Figure composited by M. Maduro from data acquired by myself, M. Maduro and G. Broitman-Maduro. Legend modified from Broitman-Maduro et al., 2009.

The next step was to determine if *ceh-51* is required for specification of coelomocytes, which is an MS-specific tissue. Since *tbx-35* mutants alone did not show to be required for coelomocyte specification, similar findings were expected for *ceh-51* mutant embryos expressing the *cup-4*::GFP marker. Thus, it was found that *ceh-51* mutants did, in fact, continue to make coelomocytes (2.1 ± 0.1, n = 53), although the numbers were significantly reduced compared to wild-type embryos (3.7 ± 0.2, n = 105, $P<10^{-12}$). Since I had previously found that *tbx-35(-); pal-1(RNAi)* embryos contained a significant reduction in the number of coelomocytes compared to wild-type embryos (0.6 ± 0.1, n = 49; 3.7 ± 0.2, n = 105, respectively), I wanted to determine whether *ceh-51(tm2123); pal-1(RNAi)* embryos also produced a lower number of *cup-4*::GFP expressing cells. It was interesting to find that *ceh-51*; *pal-1(RNAi)* embryos did not have a significant reduction in the number of coelomocytes (2.5 ± 0.1, n = 84) compared to *ceh-51* mutants alone (2.1 ± 0.1, n = 53, P = 0.01) (Table 1). Thus, *pal-1(RNAi)* does not enhance the *ceh-51*mutant phenotype (with respect to *cup-4*::GFP expression) as was the case for *tbx-35* mutant embryos. Based on the findings in *ceh-51* mutants expressing various MS-derived tissue markers, it can be concluded that CEH-51 is dispensible for specification of MS-derived tissues, but required for their normal development.

As previously described, *tbx-35* mutant embryos grown at 15ºC made as many coelomocytes as wild-type embryos, as well as more pharynx cells than mutant embryos raised at 20ºC. Also, *tbx-35* mutants grown at 15ºC elongate further than they do when grown at 20ºC. These results were unexpected since the *med-1,2* and *skn-1* mutant phenotypes are not dependent on non-permissive temperatures. Furthermore, loss of

med-1,2 results in an embryonic lethal phenotype in which arrested embryos elongate to between one and two times the length of the eggshell (Broitman-Maduro et al., 2009; Maduro et al., 2007; Maduro et al., 2001), unlike *tbx-35* mutants which arrest at variable lengths ranging from 1-fold to complete elongation and hatching (Broitman-Maduro et al., 2006). In addition to these observations, DNA-binding assays revealed TBX-35 directly binds to and activates *ceh-51* (Broitman-Maduro et al., 2009) and *ceh-51* continued to be expressed in *tbx-35* mutant embryos (Broitman-Maduro et al., 2009). Collectively, these findings suggest that a parallel factor works with TBX-35 to specify for MS-derived tissues. Therefore, it was hypothesized that *tbx-35* and *ceh-51* double mutants would result in a stronger phenotype than either single mutant alone.

To test whether the absence of both *tbx-35* and *ceh-51* resulted in a synergistic phenotype, the *tbx-35(tm1789); ceh-51(tm2123)* mutant strain was constructed using various reporters. Unlike *ceh-51* and *tbx-35* single mutant embryos, which continue to produce MS-specific tissues, *tbx-35; ceh-51* double mutants displayed a synergistic phenotype which was comparable to *med-1,2* mutant embryos. As it was seen in *med-1,2* double mutant embryos, almost no coelomocytes were detected (0.07 ± 0.03, $n = 34$), very similar to *tbx-35; ceh-51* double mutants which contained an average of 0.19 ± 0.04 coelomocytes ($n = 124$, $P<0.001$) (Table 1; Fig. 17K, L). When MS-derived pharynx cells (*pha-4*::GFP), as well as pharynx muscle, and body muscle cells (*ceh-22*::GFP and *hlh-1*::GFP, respectively), were observed in *tbx-35*; *ceh-51* double mutant embryos, similar phenotypes to those of *med-1,2* mutant embryos were also found (Fig. 17A-I) (Broitman-Maduro et al., 2009). It was previously described that *tbx-35(-)*; *mex-1*(RNAi)

embryos continue to make 1.05 ± 0.24 (n = 22) coelomocytes, suggesting that another factor is responsible for producing the coelomocytes. Therefore, *tbx-35; ceh-51* double mutant embryos were observed in a *mex-1* RNAi background and almost no coelomocytes were made (0.44 ± 0.1, n = 57, P=0.04). MS-derived pharynx muscle cells were also dramatically reduced in *tbx-35; ceh-51; mex-1* RNAi embryos expressing the *myo-2*::GFP marker (6.3 ± 0.8, n = 43), unlike *mex-1*(RNAi); *myo-2*::GFP embryos which produce over 25 pharynx muscle cells. Together, these results demonstrate that although *tbx-35* and *ceh-51* have distinct roles in the MS cell, they also have overlapping functions in specification of MS-derived tissues, since double mutant embryos exhibit a synergistic phenotype when compared to single mutant embryos.

It was previously mentioned that PAL-1 is required for specification of C- and D-derived muscle cells and is expressed in the EMS and P_2 blastomeres, however as cell divisions continue past the 24-cell stage PAL-1 is localized strictly to the P_2, C and D lineage (Hunter and Kenyon, 1996). However, in the EMS cell SKN-1 activity has been suggested to override the activity of PAL-1 (Hunter and Kenyon, 1996). In addition, *med-1,2(RNAi)* embryos MS adopts a C-like fate, producing ectopic hypodermal cells (Maduro et al., 2001). In *med-1,2* double mutants, however, *pal-1* mRNA is expressed in the MS lineage of 75% (n = 20) of embryos (Fig. 17O), while this is not detected in *med-1,2* mutant embryos rescued with the *med-1(+)* extrachromosomal array (Fig. 17M), which confirms that this C-specific regulator is responsible for the MS-to-C transformation (Broitman-Maduro et al., 2009). This transformation, however, is weaker in *tbx-35* single mutant embryos, since *pal-1* mRNA expression was detected in the MS

lineage of only a small percentage of embryos (Broitman-Maduro et al., 2006), suggesting that not all *tbx-35* mutant embryos undergo an MS-to-C transformation, unlike *med-1,2* mutant embryos. Therefore, since *tbx-35; ceh-51* double mutant embryos were showing similar mutant phenotypes to *med-1,2* double mutant embryos (dramatic decrease in production of MS-derived tissues), it was hypothesized that *tbx-35; ceh-51* mutant embryos would have a more penetrant MS-to-C transformation as was found in *med-1,2* double mutants. This, in fact, was found to be the case since 60% of *tbx-35; ceh-51* double mutant embryos expressed *pal-1* mRNA in the MS lineage (n = 35) (Fig. 17N), suggesting that the MS blastomere adopts a C-like fate in the absence of *tbx-35* and *ceh-51* (Broitman-Maduro et al., 2009).

To further confirm these findings, a C-lineage gene reporter was used, *nhr-25*::YFP, to detect hypodermal precursor cells derived from the C blastomere (Baugh et al., 2005a) in wild-type and *tbx-35; ceh-51* double mutant embryos. Since *pal-1* mRNA was found to be expressed in the MS lineage of most of the *tbx-35; ceh-51* double mutant embryos, there should ectopic expression of the *nhr-25*::YFP marker (both MS and C lineages should be expressing the marker). To determine this laser ablation was performed to ablate all cells except for the MS or C blastomeres. In wild-type embryos in which all the blastomeres were ablated except for MS, *nhr-25*::YFP was not detected (n = 3) (Fig. 18B), however the *nhr-25*::YFP reporter was detected in 5/5 isolated C blastomeres in partial wild-type embryos (Fig. 18A). As a control, the MS blastomere was also isolated in *skn-1*(RNAi) embryos expressing *nhr-25*::YFP and all embryos expressed the reporter in the isolated MS lineage (n = 3) (Fig. 18C). In addition, 9/9

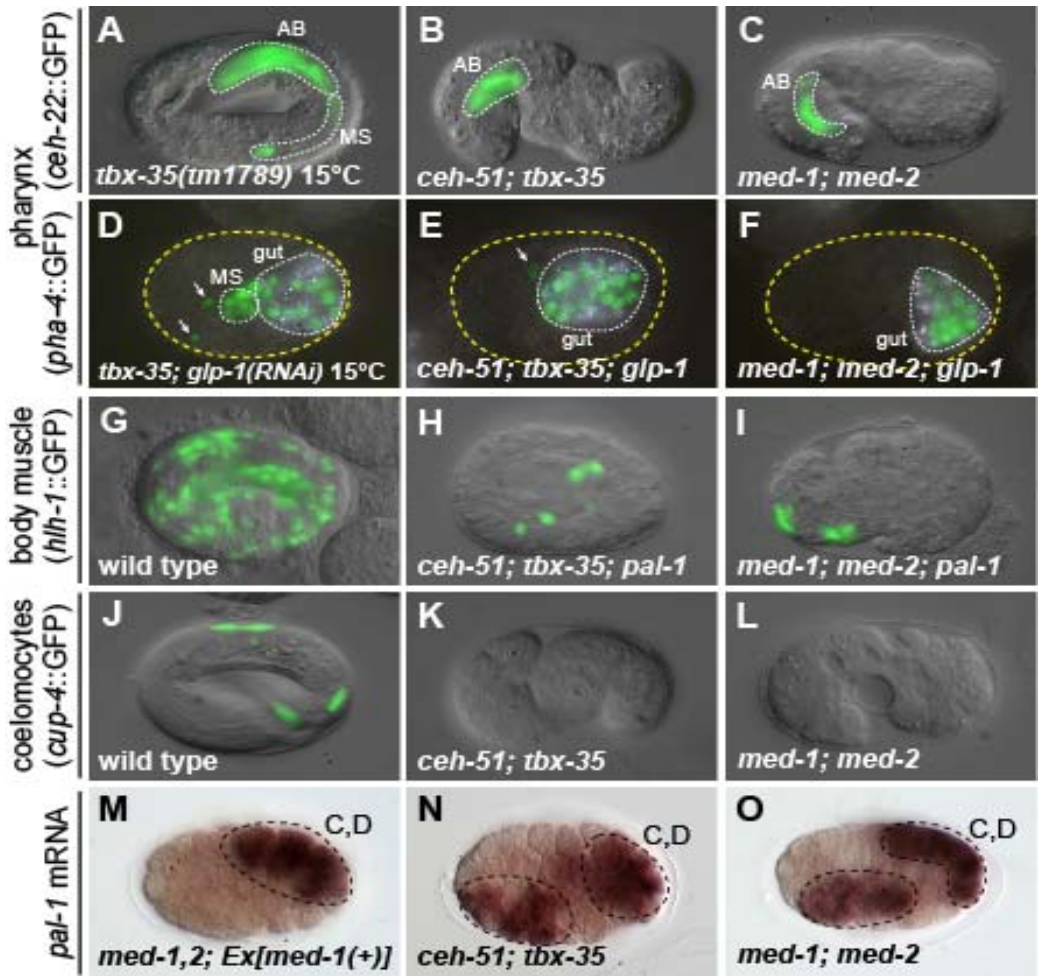


Fig. 17. Mutation of *ceh-51* and *tbx-35* together synergizes to a *med-1,2(-)* arrest phenotype. (A-C) Pharynx muscle is observed with the *ceh-22*::GFP marker (Okkema and Fire, 1994) overlaid on DIC images. (A) Arrested 1.5-fold *tbx-35(tm1789)* embryo raised at 15°C showing AB-derived and MS-derived pharynx muscles. (B) *ceh-51(tm2123); tbx-35(tm1789)* double mutant embryos arrested at ~1.5-fold elongation and only AB-derived pharynx muscle is observed. (C) *med-1(ok804); med-2(cx9744)* double mutants also showing AB-derived pharynx muscle. (D-F) Polarized light images to show gut granules overlaid with *pha-4*::GFP (Horner et al., 1998). (D) *tbx-35; glp-1(RNAi)* embryos raised at 15°C display 6.6 ± 0.5 (n = 23) pharynx cells. Some additional GFP-positive cells are seen (arrows), which is also observed in *skn-1(RNAi)* embryos (see Table 1). Gut/rectum expression of *pha-4*::GFP overlaps with birefringence of gut granules, which mark the intestine. (E) *ceh-51; tbx-35; glp-1(RNAi)* mutants show a small number of pharynx cells (arrow). (F) *med-1; med-2; glp-1(RNAi)* embryos show similar pattern as shown in E. (G-I) Body muscle cells marked by *hlh-1*::GFP (Krause et al., 1990). (J-L) Coelomocytes marked by *cup-4*::GFP (Patton et al., 2005). (M-O) In situ hybridization (performed by G. Broitman-Maduro) of *pal-1* mRNA (Baugh et al., 2005a). Expression is observed in MS descendants as well as the C and D blastomeres (N, O) (where it is normally expressed, M). Figure composited by M. Maduro from data acquired by myself, M. Maduro and G. Broitman-Maduro. Legend modified from Broitman-Maduro et al., 2009.

tbx-35; ceh-51 double mutant embryos expressed *nhr-25*::YFP reporter in the MS lineage (Fig. 18D), suggesting that a strong MS to C transformation occurs in *tbx-35; ceh-51* double mutants. Collectively, the results observed in *tbx-35; ceh-51* double mutant embryos demonstrate that CEH-51 and TBX-35 are both required for the majority of normal development of MS-specific tissues.

Discussion

The findings presented in this chapter demonstrate that even though *ceh-51*, alone, is not required for specification of MS-derived tissues it is necessary for their normal development. The *ceh-51* mutant embryos continued to make pharynx but developed abnormally based on the presence of abnormal protrusions of the pharynx as well as detached pharyngeal tissue that were observed. In addition, the *hlh-1*::GFP muscle gene reporter was used to find that in the absence of *ceh-51* the number of muscle cells produced was not significantly reduced compared with wild-type embryos; however, with the use of a different muscle marker *unc-120*::GFP additional expression of the marker was localized slightly to the posterior end of the embryo which may suggest that the absence of *ceh-51* results in migrational defects of muscle cells (Broitman-Maduro et al., 2009). Furthermore, *ceh-51* mutant embryos still produced coelomocytes (2.2 ± 0.2, $n = 47$), which also suggests that *ceh-51* is not required for specification of MS-derived tissues.

Since *tbx-35* and *ceh-51* single mutants alone did not exhibit similar phenotypes to those found in *med-1,2* mutant embryos (i.e., embryonic arrest at 1- to 2-fold stage;

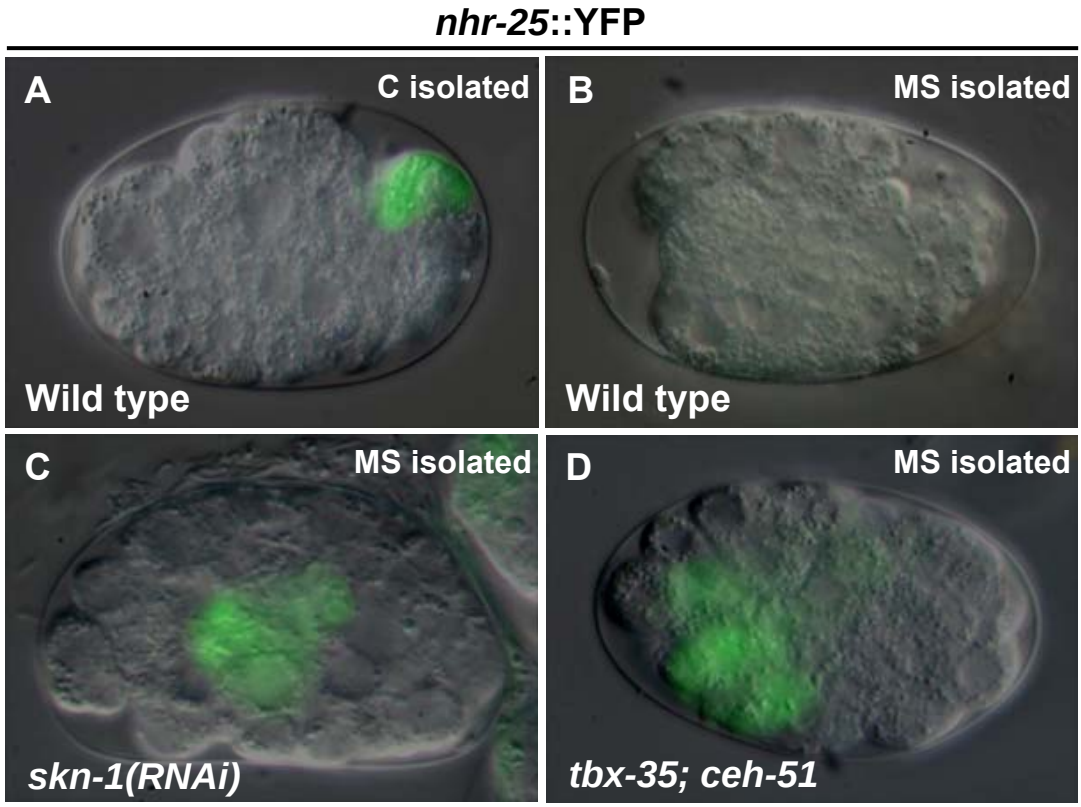


Fig. 18. Laser isolations show the MS blastomere adopts a C-like fate in *tbx-35; ceh-51* double mutant embryos. (A, B) Wild-type embryos, in which the C blastomere was isolated, displays expression of the *nhr-25*::YFP (Baugh et al., 2005a) hypodermal marker (n = 5) (A), while MS-isolated embryos do not express the *nhr-25*::YFP marker (n = 3). (C) MS-isolated *skn-1(RNAi)* embryos express the *nhr-25*::YFP marker in the region of the embryo in which MS descendants are born (n = 3). (D) Hypodermal-like cells were observed with *nhr-25*::YFP *tbx-35; ceh-51* double mutants in which the MS blastomere was isolated, suggesting that the MS blastomere adopts a C-like fate in the absence of *tbx-35* and *ceh-51*.

loss of coelomocytes, MS-derived pharynx, and body wall muscle; and internal cavities associated with ectopic hypodermal cells found in *skn-1* and *med-1,2* mutants), it was a question as to whether or not these phenotypes would be displayed in *tbx-35; ceh-51* double mutants. Although some of these characteristics were found in some *tbx-35* mutant embryos, they did not occur as often as they were observed in the *med-1,2* mutant embryos. Therefore, when *tbx-35; ceh-51* double mutant embryos were observed they did, in fact, reveal a very similar phenotype to that of *med-1,2* mutant embryos, namely strong embryonic arrest, absence of MS-derived pharynx, MS-derived body wall muscle, and coelomocytes. In addition, *tbx-35; ceh-51* double mutant embryos displayed a more penetrant MS-to-C transformation determined by laser ablation studies using the hypodermal marker, *nhr-25*::YFP.

The interesting finding, however, was that the number of coelomocytes in *ceh-51(tm2123); pal-1(RNAi)* embryos (2.5 ± 0.1, $n = 84$) was not consistent with *tbx-35; pal-1(RNAi)* double mutant embryos which contained an average of 0.6 ± 0.1 coelomocytes ($n = 49$, $P<10^{-16}$). The gene that is solely responsible for specification of embryonically-derived coelomocytes is not determined [unlike muscle tissue specification factors, HLH-1, HND-1, UNC-120, identified by Fukushige et. al. (Fukushige et al., 2006)], but it is predicted that *ceh-34* may be a coelomocyte specification factor since it has recently been found that *ceh-34* is necessary for specification of post-embryonic coelomocytes (Amin et al., 2009). Thus, we became interested to determine the expression pattern of *ceh-34* during early embryogenesis using in situ hybridization. It was found that *ceh-34* transcripts were in fact abundant in

the MS lineage and were present around the same time as *cup-4*::GFP expressing cells, which is near the "comma" stage (the stage before embryo reaches the 1-fold stage; embryo displays the shape of a comma, Fig. 4C) (G.B.M., M.O., M.M.). It has yet to be determined if *tbx-35*, *ceh-51*, or *pal-1* regulate *ceh-34* or if those genes are present long enough to regulate *ceh-34*, but based on the *tbx-35; pal-1(RNAi)* and *tbx-35; ceh-51* mutant phenotypes it is possible that *tbx-35* and *pal-1* or *tbx-35* and *ceh-51* may work together to regulate this gene, resulting in the production of coelomocytes. However, *ceh-51* and *pal-1* may not function together as *tbx-35* and *pal-1* do, providing a possible explanation for the significant reduction in the number of coelomocytes in *tbx-35; pal-1(RNAi)* embryos, and not in *ceh-51; pal-1(RNAi)* embryos. A proposed model may be that *tbx-35* binds to its binding site on the proposed *ceh-34* gene, where *ceh-51* OR *pal-1* bind to their corresponding binding sites resulting in activation of the gene and, ultimately, specification of coelomocytes. On a different note, there may be another gene, besides *ceh-34* that is responsible for specification of coelomocytes that may be downstream of *tbx-35* and *pal-1* but upstream of *ceh-51*.

Studies continue to reveal the likelihood that at least one other MS-specific activator functions upstream of *ceh-51*, since *ceh-51*::GFP was detected in some *tbx-35(tm1789)* mutant embryos. In fact, it is likely that this factor is also downstream of POP-1, since elimination of *pop-1* and *tbx-35* results in complete loss of *ceh-51*::GFP, suggesting that POP-1 may contribute to activation of *ceh-51*. Although *tbx-35* and *ceh-51* have shared and distinct functions, they may in fact have different target genes. Putative *tbx-35* binding sites have been found in *hlh-1* and *pha-4* promoter region

(W.W.K.H, M.F.M, unpublished), but further studies are required to confirm this. Whether or not *ceh-51* binding sites exist, however, is still not known. Therefore to further expand on the mesoderm gene regulatory network, it would be essential to explore and elucidate the downstream target genes of *tbx-35* and *ceh-51*.

It has been a common finding in other species that both TBX and NK-2 class homeodomain transcription factors interact to give rise to mesoderm tissues, for example cardiac tissues, which is comparable to *C. elegans* pharynx. In fact, it has been found that in *Drosophila*, the specification of cardiac fate is complex; meaning that more than one factor is usually required to induce mesoderm fate and ultimately give rise to cardiac tissues (Brody, 2005). In *Drosophila* mutations in the *Nkx2.5* ortholog, *tinman*, result in abnormal development of aspects of the heart, visceral muscle and some body muscles, suggesting that the gene is necessary for proper development of these mesoderm tissues (Azpiazu and Frasch, 1993; Bodmer, 1993). In mice, *Tbx5* and *Nkx2.5* has been found to directly interact and work together with *Gata4/5* to activate cardiac genes (Hiroi et al., 2001; Stennard et al., 2003). It was also recently reported that the GATA transcription factor, *pannier* (*pnr*), is a critical regulator of heart progenitor formation in *Drosophila* (Qian and Bodmer, 2009). In addition, *Tbx20/neuromancer* (*nmr*), is a downstream mediator of *pnr* and together with *Nkx2.5* ortholog, *tinman*, establish early cardiac progenitor formation and maintain heart function (Qian and Bodmer, 2009). These interactions between GATA, T-BOX, and NK-2 class factors and their role in specification of mesoderm fate are also found in *C. elegans* embryonic development, as reported in the text, suggesting that the function of these factors have been shown to be

conserved. As mentioned earlier, the NK-2 class factors (i.e., *ceh-34*, *ceh-51*) are a subfamily of the homeobox genes which are conserved in metazoans. Also, coelomocytes are blood-like cells that act as scavengers, meaning that they uptake foreign substances within the *C. elegans* body cavity, similar to macrophage white blood cells. It has recently been reported that an NK-like homoebox gene, *msx2*, is involved in mediating the activation of NOTCH3 signaling pathway responsible for T-cell differentiation in leukemic cell lines (Nagel et al., 2009). Therefore, it is possible that a similar mechanism exists in *C. elegans* in which *ceh-34* positively regulates Notch signaling (which occurs between ABa and MS) for differentiation of coelomocyte precursor cells. In conclusion, further experiments designed to elucidate the gene network downstream of TBX-35 and CEH-51 may provide more insight into conserved aspects of cardiac and mesoderm development.

Chapter 4: The role of the Wnt effector POP-1/TCF in the *C. elegans* endomesoderm specification gene network

The role of the Wnt effector, TCF/POP-1: making MS and E different

At the four-cell stage of the *C. elegans* embryo, the P_2 blastomere sends a Wnt signal to the posterior end of the EMS cell (Fig. 9). The Wnt signal induces the β-catenin-like factor, WRM-1, to activate the Nemo-like kinase, LIT-1, and forms a complex to phosphorylate POP-1 and export it out of the nucleus (Rocheleau et al., 1999). This results in lower nuclear levels of POP-1 in the posterior daughter cell of EMS, while POP-1 nuclear levels remain high in the EMS anterior daughter cell. Meanwhile, another β-catenin factor, SYS-1, binds to the remaining POP-1 factor in the nucleus to activate transcription of endoderm specifying genes, *end-1* and *end-3*. Thus, the E blastomere is specified as a result of the Wnt signaling pathway. On the other hand, in the anterior EMS daughter cell, MS, the nuclear levels of POP-1 remain high and acts as a transcriptional repressor, along with its co-repressors, to block transcription of *end-1* and *end-3*. Thus, mesoderm-specifying genes are activated through the SKN-1 regulatory pathway and the MS cell is specified. If the EMS cell is isolated from the embryo at the four-cell stage, the cell will divide and give rise to two MS-like cells (Goldstein, 1993). In addition if the P_2 cell is placed on the anterior end of EMS then the anterior daughter cell will result in specification of the E fate, while the posterior daughter cell becomes MS (Goldstein, 1993). Thus the P_2-EMS interaction is responsible for establishing the MS and E asymmetry.

Furthermore, POP-1 has been shown to play a role in proper establishment of the proximal/distal polarity of the somatic gonad precursor cells (Asahina et al., 2006). In the absence of POP-1 or any of its upstream factors (i.e., WRM-1, SYS-1, etc.), all the daughters of the somatic gonad precursor cells adopt the proximal fate, resulting in failure of the gonad to develop properly (Asahina et al., 2006). Therefore, POP-1 is an essential factor in regulating asymmetric cell divisions resulting in proper cell fate specification. Besides it's positive role in specification of the E blastomere, it was formerly suggested that POP-1 also plays a role in MS specification (Lin et al., 1995). In this chapter, however, the role of POP-1 in the MS cell of *C. elegans* is investigated further, by silencing *pop-1* via RNAi in an *end-1*, *end-3* mutant background. Blocking the activity of the endoderm specifying genes in a *pop*-1 mutant background will provide a more direct assessment of POP-1 in MS specification, since it is the activation of *end-1* and *end-3* that results in the E fate to arise.

Ectopic MS-derived tissues are made in end-3; pop-1 and end-1,3; pop-1 embryos

In light of the fact that the MS-specific genes, *tbx-35* and *ceh-51*, continue to be expressed in the MS and E lineages in the absence of POP-1, I was interested to determine if POP-1 may have an additional role in the MS blastomere besides repression of the endoderm specifying genes *end-1* and *end-3*. It was also found that POP-1 has a positive role in specifying the MS fate in parallel with SKN-1 in the related nematode *C. briggsae* (Lin et al., 2009). In addition, it was previously shown by a different group of researchers that POP-1 had a positive role in MS specification (Lin et al., 1995).

As previously described, an E-to-C-like transformation occurs in the absence of *end-1* and *end-3* genes (Maduro et al., 2005a). However, in *pop-1* mutant embryos, the MS blastomere adopts the E fate. Thus, it was predicted that in the absence of *pop-1*, *end-1*, and *end-3* the MS and E blastomeres will adopt a C-like fate. The question was, whether POP-1 is required in MS for more than just repression of *end-1* and *end-3* in the MS cell. Therefore, I was interested to determine what effect the absence of *end-1* and *end-3* would have on the fate of the E blastomere in combination with *pop-1* being absent. Initially these experiments began with strains that were comprised of various MS-specific markers (i.e., *cup-4*::GFP, *myo-2*::GFP, *pha-4*::GFP, *unc-120*::GFP, etc.) and an *end-3* allele (*ok1448*), obtained from the *C. elegans* Gene Knockout Consortium, in which its DNA-binding domain was deleted. The *ok1448* allele is a putative null allele because it deletes the coding region for the END-3 DNA-binding domain. Although it is known that POP-1 activates the endoderm genes, *end-1* and *end-3*, in the E blastomere (Shetty et al., 2005), studies have also reported that *end-3* mRNA transcripts appear earlier than *end-1* mRNA, and the majority of *end-3; pop-1* double mutant embryos (~97%) lack endoderm (Maduro et al., 2005a) suggesting that END-3 also plays a role in activating *end-1* (Maduro et al., 2007). Therefore, the preliminary experiments consist of observations made from *end-3; pop-1(zu189)* or *end-3; pop-1(RNAi)* mutant embryos using MS-specific markers.

Ultimately, a chromosomal *end-1(ok558)*, *end-3(ok1448)* double mutant became available and the experiments were repeated in this background [*end-1,3; pop-1(RNAi)* or *end-1,3; pop-1(zu189)*]. The *ok558* allele is a putative null allele that contains a partial

deletion of the DNA-binding domain, thus affecting its binding with transcription factors (Maduro et al., 2005a). It has been shown that *pop-1* is expressed maternally and zygotically (Lin et al., 1998; Lin et al., 1995). The *zu189* allele is considered a putative null allele for *pop-1* because it consists of an insertion of a transposable element in the 3'-UTR of the *pop-1* gene, thus affecting the stability of the maternal mRNA (Lin et al., 1998; Lin et al., 1995). Thus, the *pop-1(zu189)* mutation affects the maternal expression of *pop-1* and was identified in a genetic screen for maternal effect lethal mutants with defects in pharyngeal development (Lin et al., 1995). On the other hand, *pop-1(RNAi)* reduces both maternal and zygotic expression resulting in a more severe phenotype compared to *pop-1(zu189)* embryos (Lin et al., 1998). Therefore, both methods of down-regulating *pop-1* were used to examine any possible differences in specification of pharynx muscle progenitors.

Anterior pharynx tissue is only one of many tissue types made by the MS blastomere. As previously described, the MS cell produces anterior pharynx, body wall muscle and four embryonically-derived coelomocytes. Therefore, all of these tissue types were observed in *pop-1*, *end-1*, *end-3* mutant embryos. The initial observations were made using the *cup-4*::GFP coelomocyte marker in *end-3(ok1448); pop-1(RNAi)* mutant backgrounds. These embryos produced an average of 6.1 ± 0.2 coelomocytes (n = 77), which is a significant increase in production of coelomocytes compared to wild-type embryos (3.7 ± 0.2, n = 105, $P<10^{-14}$) (Table 2; Fig. 19A, E). Since the MS blastomere adopts an E-like fate in *pop-1(RNAi)* embryos expression of *cup-4*::GFP was

Table 2. Coelomocytes and pharynx cells made in wild-type, mutant and RNAi backgrounds

Genotype	coelomocytes *cup-4*::GFP[1]	pharynx cells *pha-4*::GFP[1,2]
wild type (N2)	3.7 ± 0.2 (105)	50.0 ± 0.9 (21)
pop-1(RNAi)	0.0 ± 0.0 (50)	21.3 ± 0.8 (18)
glp-1(RNAi)	nd	23.1 ± 0.6 (15)
end-1(ok558) end-3(ok1448)	4.5 ± 0.1 (65)	49.1 ± 1.0 (10)
end-1(ok558) end-3(zu247)	4.6 ± 0.1 (77)	51.1 ± 1.3 (15)
end-3(ok1448); pop-1(RNAi)	6.1 ± 0.2 (77)	28.5 ± 7.0 (22)
end-1(ok558) end-3(zu247); pop-1(RNAi)	9.3 ± 0.2 (80)	43.5 ± 1.3 (23)
end-1(ok558) end-3(ok1448); pop-1(RNAi)	10.7 ± 0.3 (77)	44.8 ± 2.2 (17)
end-3(ok1448); glp-1(or178)	nd	21.2 ± 0.8 (18)
end-3(ok1448); glp-1(or178); pop-1(RNAi)	nd	9.5 ± 0.8 (75)
end-1(ok558) end-3(ok1448); glp-1(or178)	nd	25.2 ± 1.0 (17)
end-1(ok558) end-3(ok1448); glp-1(or178); pop-1(RNAi)	nd	16.8 ± 1.1 (93)
end-1(ok558) end-3(ok1448); glp-1(or178); pop-1(RNAi); Ex[end-1,3(+)]	nd	1.4 ± 0.3 (40)
med-1(ok804); med-2(cx9744)	0.1 ± 0.0 (34)	31.3 ± 0.6 (26)
ceh-51(tm2123); tbx-35(tm1789)	0.2 ± 0.0 (124)	30.2 ± 0.5 (44)
med-1(ok804); med-2(cx9744); end-1(ok558) end-3(ok1448)[3]	0.0 ± 0.0 (52)	29.9 ± 1.1 (12)
med-1(ok804); med-2(cx9744); end-1(ok558) end-3(ok1448); pop-1(RNAi)[3]	1.0 ± 0.2 (57)	22.4 ± 0.9 (24)
end-1(ok558) end-3(ok1448) ceh-51(tm2123); tbx-35(tm1789)	0.1 ± 0.0 (122)	30.3 ± 1.0 (21)
end-1(ok558) end-3(ok1448) ceh-51(tm2123); tbx-35(tm1789); pop-1(RNAi)	0.1 ± 0.1 (35)	22.1 ± 1.4 (19)

[1]Some data are from Broitman-Maduro et al. 2009.
[2]Expression of *pha-4* in gut and/or rectum cells (if present) was not included.
[3]Embryos were also homozygous for *dpy-11(e224)*.
Table composited by M. Maduro from data acquired by myself, M. Maduro, and G. Broitman-Maduro (Owraghi et al., 2009)

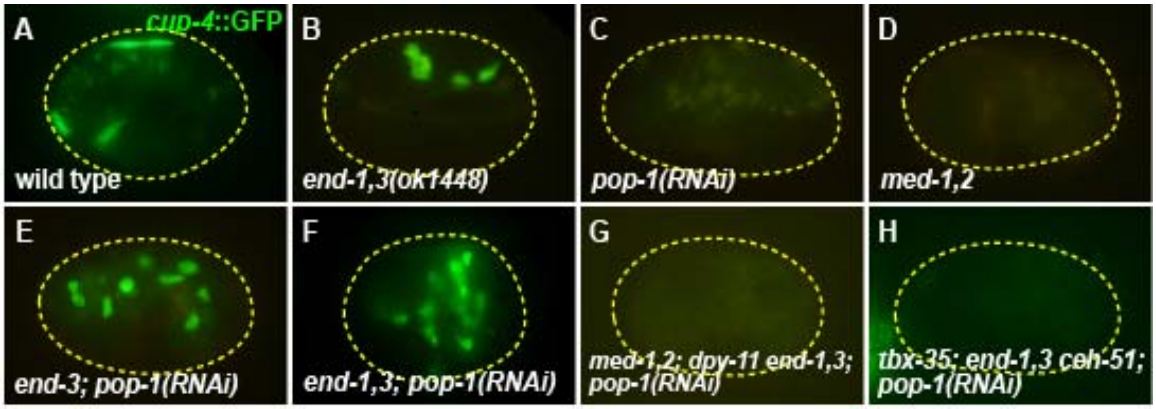


Fig. 19. Coelomocytes are made in *end-1,3; pop-1* embryos. All embryos carry a *cup-4*::GFP coelomocyte-specific marker (Patton et al., 2005). (A) Wild-type emrbyo showing accumulation of *cup-4*::GFP expression in four coelomocytes (the top two are adjacent). (B) Normal production of coelomocytes in an *end-1(ok558); end-3(ok1448)* mutant. (C, D) Loss of coelomocytes in *pop-1(RNAi)* and *med-1(ok804); med-2(cx9744)* embryos, in which a penetrant mis-specification of MS occurs (Broitman-Maduro et al., 2009; Lin et al., 1995; Maduro et al., 2001). (E, F) Ectopic coelomocytes are made in *end-3(ok1448); pop-1(RNAi)* embryos (average of 6.1 ± 0.2 *cup-4*::GFP expressing cells, n = 77) and *end-1(ok558); end-3(ok1448); pop-1(RNAi)* embryos (average of 10.7 ± 0.3 cells, n = 77). (G, H) Loss of coelomocytes in *med-1(ok804); med-2(cx9744); dpy-11(e224) end-1(ok558) end-3(ok1448); pop-1(RNAi)* embryos and *tbx-35(tm1789); ceh-51(tm2123) end-1(ok558) end-3(ok1448); pop-1(RNAi*) embryos. Faint yellow signal in some panels corresponds to gut granules. Figure composited by M. Maduro from data acquired by myself. Legend modified from Broitman-Maduro et al., 2009.

observed in these embryos as a control. As expected, no coelomocytes were detected (n = 50) (Fig. 19C). These experiments were repeated in *end-1(ok558) end-3(ok1448); pop-1(RNAi)* embryos. The phenotype observed in *end-3*; *pop-1(RNAi)* embryos was enhanced in *end-1(ok558) end-3(ok1448); pop-1(RNAi)* embryos in which an average of 9.3 ± 0.2 coelomocytes were made (n = 80) (Table 2; Fig. 19F).

In addition, *end-1(ok558) end-3(zu247); pop-1(RNAi)* embryos were shown to have extra coelomocytes, with embryos producing an average of 10.7 ± 0.3 coelomocytes (n = 77), suggesting that the type of *end-3* mutation (whether it is a deletion of the DNA-binding domain or a point mutation) does not make a difference in the phenotype and that both result in detrimental defects in the embryo (i.e., lack of endoderm). In *end-1(ok558) end-3(ok1448)* [will be referred to as *end-1,3(ok1448)*; *end-1(ok558) end-3(ok1448)* as *end-1,3(zu247)*] mutant embryos normal number of coelomocytes should be made, therefore these embryos were observed, as a control, and they were found to produce an average of 4.5 ± 0.1 (n = 65) (Table 2; Fig. 19B), as well as *end-1,3(zu247)* double mutants which produced an average of 4.6 ± 0.1 coelomocytes (n = 77) (Owraghi et al., 2009). These sets of data suggest that in the absence of *end-1*, *end-3*, and *pop-1* MS and/or other cell(s) may be producing extra coelomocytes.

It was of interest to next determine if other MS-specific tissues are also produced ectopically in the absence of *end-1*, *end-3* and *pop-1*. To score the number of MS-derived pharynx cells, the *pha-4*::GFP marker was used (Horner et al., 1998). The *pha-4*::GFP reporter marks the pharynx, digestive tract, gut, and hindgut (rectum). These cells can be differentially identified by the size of the nucleus and location of the cells or

by using other methods to selectively eliminate non-MS derived sources of *pha-4*::GFP expression. In wild-type embryos, an average of 50.0 ± 0.1 pharynx cells are made (n = 21). With *pop-1(RNAi)*, embryos made 21.3 ± 0.8 cells (n = 18), which is consistent with the fact that these cells are ABa-derived and that MS-derived pharynx cells are lost due to the MS-to-E transformation. Slightly more pharynx cells were produced, however, in *end-3(ok1448); pop-1(RNAi)* embryos (28.5 ± 7.0, n = 22) when compared to *pop-1(RNAi)* embryos (21.3 ± 0.8 , n = 18) (Table 2; Fig. 20C), while *end-1,3(ok1448); pop-1(RNAi)* and *end-1,3(zu247); pop-1(RNAi)* embryos made 44.8 ± 2.2 (n =17) (Table 2; Fig. 20G) and 43.5 ± 1.3 (n = 23) pharynx cells, respectively (Owraghi et al., 2009). It is not known that the additional *pha-4*::GFP(+) cells are produced by MS or are just the result of extra cell divisions within the AB lineage.

The AB cells which normally make neurons and hypodermal cells in isolation, has been shown to make pharyngeal cells, as well (Hutter and Schnabel, 1994; Sulston et al., 1983). The production of anterior pharynx from the ABa lineage has been shown to be the result of interactions between the ABa and MS cells which involves the GLP-1/Notch-like receptor in the Notch signaling pathway (Fig. 10B) (Mello et al., 1994; Priess et al., 1987; Priess and Thomson, 1987). Embryos in which *glp-1* has been knocked-down via RNAi, exhibit less pharynx cells (23.1 ± 0.6, n = 15) (Table 2), when compared to wild-type embryos which express an average of 50.0 ± 0.9 pharynx cells (n = 21). These results are consistent with previous findings that ABa-derived pharynx is *glp-1*-dependent (Priess et al., 1987). With this in mind, a strain was constructed in which the *glp-1(or178)* mutation was incorporated into the *end-3(ok1448); pop-1(RNAi)*

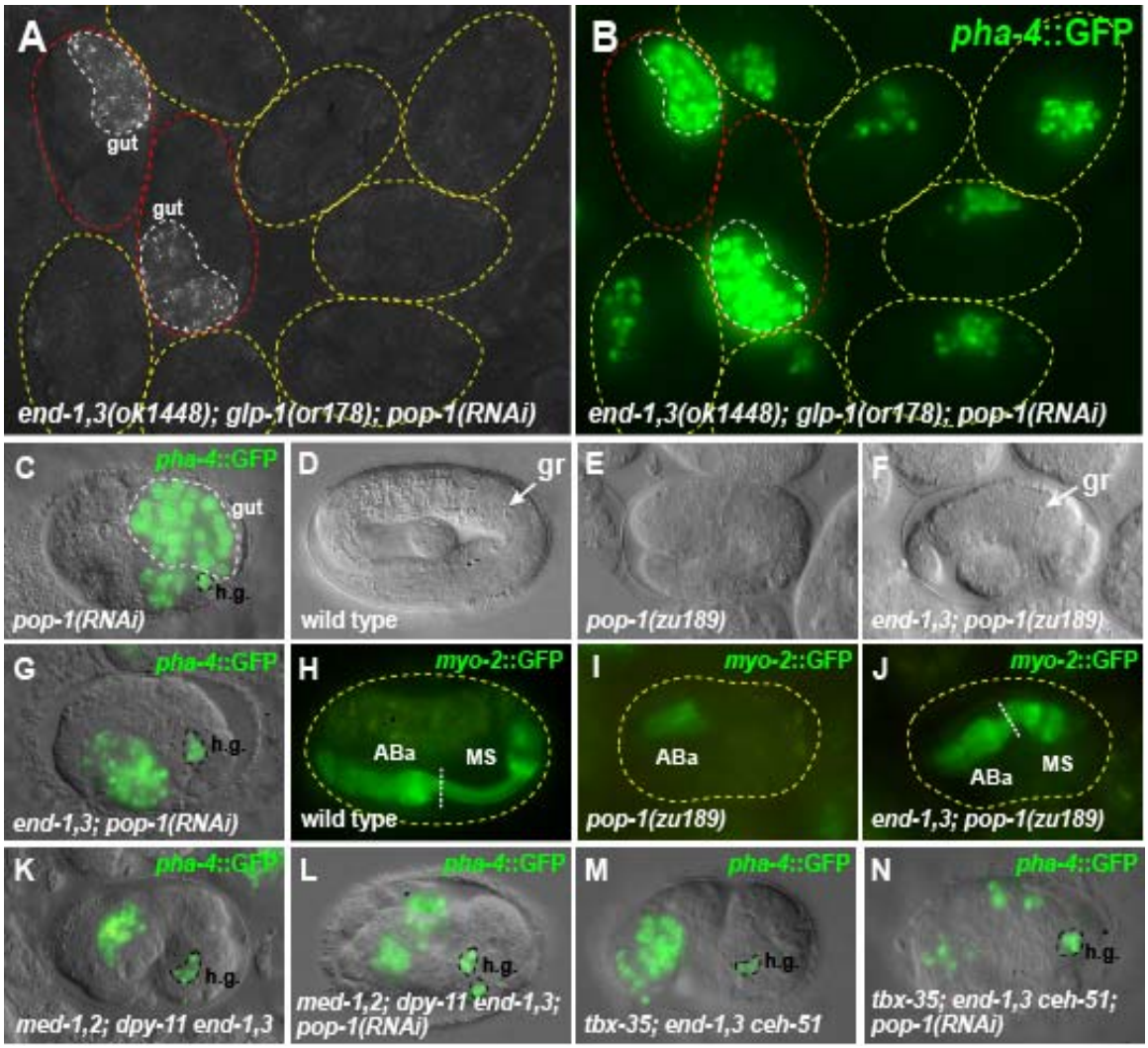
and *end-1,3(ok1448); pop-1(RNAi)* mutant embryos and MS-derived pharynx cells were scored. As expected, in the control embryos, *end-3(ok1448); glp-1(or178),* and *end-1,3(ok1448); glp-1(or178)* an average of 21.2 ± 0.8 (n = 18) and 25.2 ± 1.0 (n = 17) pharynx cells were produced, respectively, which are comparable to the number of MS-derived pharynx cells produced in *glp-1(RNAi)* embryos (Owraghi et al., 2009). However, *end-3(ok1448); glp-1(or178); pop-1(RNAi)* embryos made an average of 9.5 ± 0.8 cells (n = 75) and *end-1,3(ok1448); glp-1(or178); pop-1(RNAi)* embryos produced 16.8 ± 1.1 cells (n = 93) (Table 2; Fig. 20A, B) (Owraghi et al., 2009). In addition, *end-1,3(ok1448); glp-1(or178); pop-1(RNAi)* embryos that were rescued with an *end-1,3(+)* extrachromosomal transgene array, *Ex*[*end-1,3(+)*], did not produce many pharynx cells (1.4 ± 0.3 cells, n = 40) (Table 2; Fig. 20A, B), consistent with the loss of both AB- and MS-derived pharynx cells in *glp-1; pop-1* mutant embryos (Lin et al., 1995; Owraghi et al., 2009). Collectively, the data suggests that GLP-1-independent pharynx cells are restored in both *end-3(ok1448); pop-1(RNAi)* and *end-1,3(ok1448); pop-1(RNAi)* embryos.

To determine whether or not the pharynx cells restored to *end-1,3; pop-1* embryos undergo organogenesis, experiments were conducted in *end-1,3(ok1448); pop-1(zu189)* triple mutant embryos mutant embryos expressing the pharynx muscle reporter, *myo-2*::GFP. Wild-type embryos make a complete pharynx along with a grinder (Fig. 20H) [a cuticle specialization made by a subset of pharynx muscle cells that rotate when the muscle contracts; similar to 'teeth' that grind up the food (Altun, 2008)]. A partial grinder was detected in approximately 23% of the triple mutant embryos (n = 74) **(**Fig.

20J**)**. Mutant embryos, however, that are rescued for *end-1,3* [express the *end-1,3*(+) transgene array] never show development of a grinder, similarly found in *pop-1(RNAi)* embryos (Fig. 20I) (Owraghi et al., 2009). Therefore, the additional pharynx cells made in *end-1,3; pop-1(zu189)* embryos can sometimes undergo organogenesis to generate part of the posterior pharynx. In addition, this is further evidence that the pharynx cells restored to the MS lineage are similar to those made by MS.

The next step was to examine the origin of the extra coelomocytes and pharynx cells found in *end(-); pop-1(-)* embryos by performing laser ablation studies. These mutant embryos contained either the *cup-4*::GFP marker, *myo-2*::GFP which marks pharynx muscle (Okkema et al., 1993), or the body wall muscle reporter, *unc-120*::GFP (Fukushige et al., 2006). When ABa, MS, and E blastomeres were ablated in *end-3(ok1448); pop-1(zu189)* and *end-1,3(ok1448); pop-1(zu189)* embryos expressing the *myo-2*::GFP marker, no pharynx muscle was detected (n = 3 for each genotype). Ablation of only ABa + MS or ABa + E, however, resulted in production of pharynx muscle in partial embryos; although the expression of *myo-2* in ABa + MS ablated *end-3(ok1448); pop-1(zu189)* embryos was somewhat weaker than in *end-1,3(ok1448); pop-1(zu189)* embryos (Table 3). Individual cells could not be distinguished, since the *myo-2* reporter that was used was localized cytoplasmically, thus the number of cells could not be counted. A similar experiment was conducted in which ABa + ABp + MS or E were ablated in *end-1,3(ok1448); pop-1(RNAi)* embryos. The findings were slightly different in that 4/6 ABa + ABp + E ablated embryos and 5/8 ABa + ABp + MS ablated embryos

Fig. 20. Embryos lacking *end* and *pop-1* function make MS-type pharynx. (A) Polarized light image of a field of *end-1(ok558) end-3(ok1448); glp-1(or178ts)* embryos raised at the non-permissive temperautre for *or178* and treated with *pop-1(RNAi)*. The two embryos on the left (outlined in red) carry an *end-13(+)* transgene and are the only embryos that produced gut. (B) Fluorescence image of the same embryos in panel A showing *pha-4*::GFP expression (Horner et al., 1998). Expression in the two rescued embryos (red outline) is from gut cells, while in the remaining embyos expression is from GLP-1-independent pharynx cells (an average of 16.8 ± 1.1 cells, n = 93). (C) GFP overlay of *pha-4*::GFP expression in a *pop-1(RNAi)* embryos. The bright signals in large nuclei are gut nuclei (confirmed by polarized light birefringence), while the remaining expression is from hindgut cells (h.g.) and ABa-derived pharynx (average of 21.3 ± 0.8 cells, n = 18). (D) Elongated wild-type embryo with grinder (gr) indicated. (E) Arrested *pop-1(zu189) dpy5(e61)* embryo from *pop-1 dpy-5* mother. (F) Arrested *end-1(ok558) end-3(ok1448); pop-1(zu189) dpy-5(e61)* embryo from an *end-1,3; pop-1 dpy-5* mother rescued by an *end-1,3(+)* transgene. The grinder (gr) within a posterior pharynx-like structure is indicated. (G) Expression of *pha-4*::GFP in an *end-1(ok558) end-13(ok1448); pop-1(RNAi)* embryo in hindgut (h.g.) and pharynx (average of 44.8 ± 2.2 pharynx cells, n = 17). (H-J) *myo-2*::GFP expression (Okkema et al., 1993) showing presence of ABa- and MS-derived pharynx in wild-type (H) and *end-1,3; pop-1* (J) embryos, but lacking MS0derived pharynx in a *pop-1* mutant (I). (K, L) pha-4::GFP expression observed in *med-1(ok804); med-2(cx9744); dpy-11(e224) end-1(ok558) end-3(ok1448*) background. (M, N) The number of *pha-4*::GFP expressing cells is reduced from ~30 in *tbx-35(tm1789); end-1(ok558) end-3(ok1448) ceh-51(tm2123)* embryos to ~22 cells when *pop-1(RNAi)* is added. Embryos in images have been oriented with anterior to the left and dorsal up. Figure composited by M. Maduro from data acquired by M. Maduro and G. Broitman-Maduro. Legend modified from Broitman-Maduro et al., 2009.


A
gut
gut
end-1,3(ok1448); glp-1(or178); pop-1(RNAi)
B
pha-4::GFP
end-1,3(ok1448); glp-1(or178); pop-1(RNAi)
C
pha-4::GFP
gut
h.g.
pop-1(RNAi)
D
gr
wild type
E
pop-1(zu189)
F
gr
end-1,3; pop-1(zu189)
G
pha-4::GFP
h.g.
end-1,3; pop-1(RNAi)
H
myo-2::GFP
ABa
MS
wild type
I
myo-2::GFP
ABa
pop-1(zu189)
J
myo-2::GFP
ABa
MS
end-1,3; pop-1(zu189)
K
pha-4::GFP
h.g.
med-1,2; dpy-11 end-1,3
L
pha-4::GFP
h.g.
med-1,2; dpy-11 end-1,3; pop-1(RNAi)
M
pha-4::GFP
h.g.
tbx-35; end-1,3 ceh-51
N
pha-4::GFP
h.g.
tbx-35; end-1,3 ceh-51; pop-1(RNAi)

expressed *myo-2*::GFP (Fig. 21A, B; Table 3). Collectively, the results suggest that both MS and E can produce pharynx muscle in *end-3; pop-1* and *end-1,3; pop-1* embryos. The difference in the outcome of the two genotypes is most likely a result of the different effects of *pop-1(zu189)* vs. *pop-1(RNAi)*. Analysis has been previously done on the AB lineage in *pop-1(zu189)* and *pop-1(RNAi)* embryos and it is reported that in *pop-1(zu189)* embryos, POP-1 expression is not detected in ABxxa/ABxxp, but is expressed in subsequent descendants (Lin et al., 1998). This result is consistent with the observation that in *pop-1(zu189)* embryos, ABxxa adopts the fate of ABxxp, but the subsequent a/p asymmetries remain normal in the lineage (Lin et al., 1998). However, the same analysis was done in *pop-1(RNAi)* embryos and it was found that POP-1 was not detected in ABxxa/ABxxp blastomeres or in its descendants (Lin et al., 1998). It was found that in these embryos the anterior fates in multiple, sequential a/p decisions failed to become specified (Lin et al., 1998). These findings demonstrate that maternal-specific depletion of *pop-1* can result in an anterior-to-posterior fate transformation only early on in the developmental process while the descendants undergo normal asymmetric division; whereas depletion of both maternal and zygotic *pop-1* results in an anterior-to-posterior fate transformation throughout the embryo, resulting in all of the cells adopting a posterior fate. Since pharynx tissue is made by the anterior blastomeres (descendants of ABa and descendants of MSa), depletion of maternal and zygotic *pop-1*, as well as *end-3* or *end-1,3*, is expected to result in no pharynx produced, although may arise some of the time depending on how effective the reduction of *pop-1* activity is in subsequent cell

divisions, or how stringent the requirement for POP-1 is in asymmetric divisions later in the lineage (Fig. 22).

Table 3. Production of tissues in laser-operated embryos

Genotype	Blastomeres ablated or isolated	Partial embryos making tissues (# of cells) coelomocytes (*cup-4*::GFP)	pharynx muscle (*myo-2*::GFP)[1]	body muscle (*unc-120*::GFP)	epidermis (*nhr-25*::YFP)[1]
wild type	MS abl.	0/3 (0.0 ± 0.0)	-	-	-
	MS iso.	3/3 (4.0 ± 0.0)	-	-	0/1
	E iso.	0/6 (0.0 ± 0.0)	-	0/4 (0.0 ± 0.0)	0/6
	C iso.	-	-	-	5/5
skn-1(RNAi)	MS iso.	-	-	-	3/3
end-3(ok1448)	MS abl.	0/5 (0.0 ± 0.0)	-	-	-
	E abl.	2/2 (4.0 ± 0.0)	-	-	-
end-3(ok1448); pop-1(zu189)[2]	ABa+MS abl.	-	3/3 (weak)	-	-
	ABa+E abl.	-	2/2	-	-
	ABa+MS+E abl.	-	0/3	-	-
end-3(ok1448); pop-1(RNAi)	MS abl.	4/4 (3.0 ± 0.6)	-	-	-
	E abl.	5/5 (3.0 ± 0.5)	-	-	-
	MS+E abl.	1/4 (0.3 ± 0.2)	-	-	-
end-1(ok558) end-3(ok1448)	MS abl.	1/5 (0.2 ± 0.2)	-	-	-
	E abl.	5/5 (3.6 ± 0.2)	-	-	-
	MS+E abl.	0/3 (0.0 ± 0.0)	-	-	-
	MS iso.	5/5 (4.0 ± 0.0)[3]	-	3/3 (18.7 ± 2.0)	0/5[3]
	E iso.	0/4 (0.0 ± 0.0)[3]	-	3/3 (15.3 ± 0.7)	10/10[3]
pop-1(zu189)[2]	MS abl.	-	1/1	-	-
	ABa abl.	-	0/3	-	-
pop-1(RNAi)	MS iso.	-	-	-	0/3
	E iso.	-	-	-	0/4
end-1(ok558) end-3(ok1448); pop-1(zu189)[2]	ABa+MS abl.	-	5/5	-	-
	ABa+E abl.	-	4/4	-	-
	ABa+MS+E	-	0/3	-	-
end-1(ok558) end-3(ok1448); pop-1(RNAi)	MS abl.	3/3 (6.7 ± 0.5)	-	-	-
	E abl.	3/3 (4.7 ± 0.7)	-	-	-
	MS+E abl.	0/4 (0.0 ± 0.0)	-	-	-
	MS iso.	4/4 (5.0 ± 0.4)	-	-	0/8
	E iso.	6/6 (5.5 ± 0.3)	-	6/6 (10.2 ± 1.2)	9/11
	ABx+MS abl.	-	5/8	-	-
	ABx+E abl.	-	4/6	-	-

[1]Numbers of cells were not counted because individual cells could not be distinguished.
[2]Embryos from homozygous *pop-1(zu189) dpy-5(e61)* mothers (with other mutations as indicated).
[3]Numbers from experiments on a strain carrying both *nhr-25*::YFP and *cup-4*::GFP were included.
Abbreviations: abl, ablated; iso, isolated (all other blastomeres ablated); ABx, both daughters of AB were ablated. **Table composited by M. Maduro from data acquired by myself (Owraghi et al., 2009).**

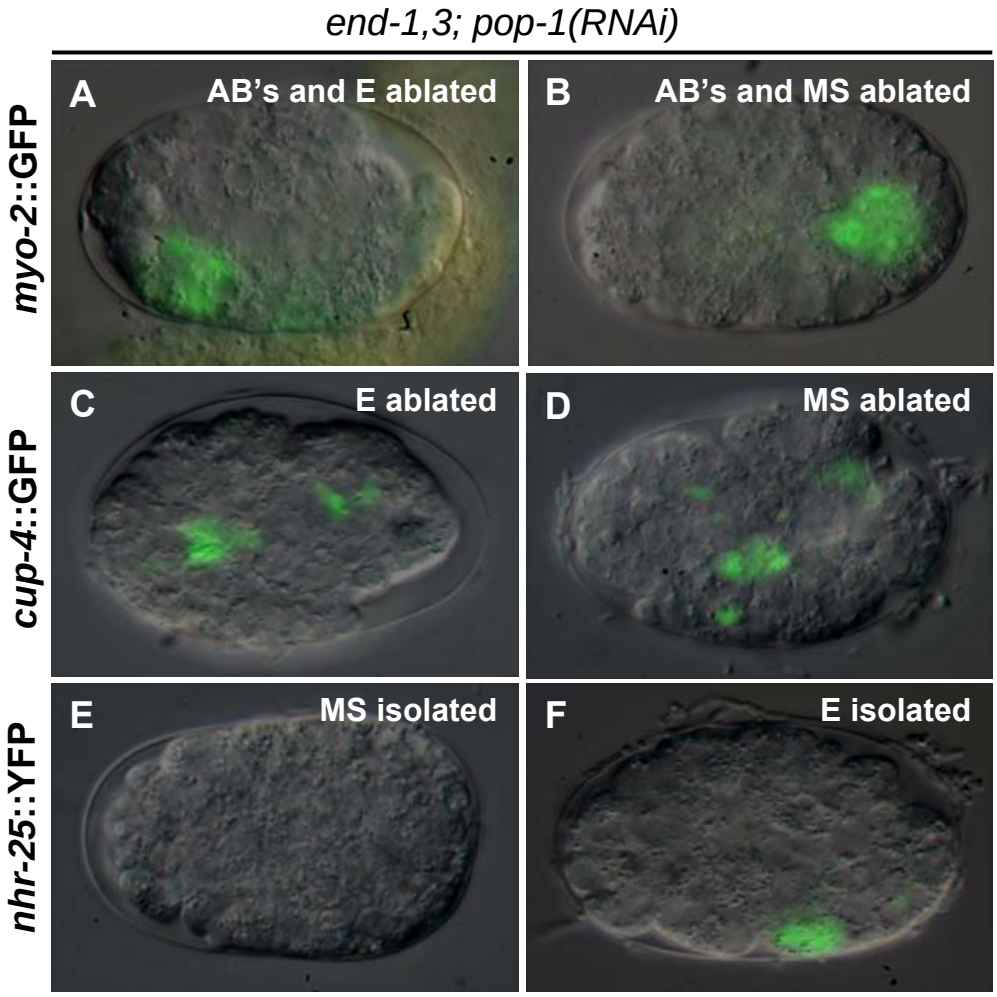


Fig. 21. The E blastomere in laser ablated *end-1,3; pop-1(RNAi)* embryos display expression of MS- and C-specific markers. (A) In four out of six ABa, ABp, E ablated, triple mutant embryos, the MS descendants express the *myo-2*:GFP (Okkema, 1993) marker, while five out of eight of the E descendants in the ABa, ABp, MS ablated embryos express the *myo-2*::GFP (B). (C) Triple mutant embryos, in which the E blastomere was ablated, express MS-derived *cup-4*::GFP (average of 4.7 ± 0.7 *cup-4*::GFP expressing cells, n =3). (D) The E descendants in MS ablated, triple mutant embryos continued to make coelomocytes expressing an average of 6.7 ± 0.5 *cup-4*::GFP expressing cells (n = 3). (E) The hypodermal marker, *nhr-25*::YFP (Baugh et al., 2005a), was not detected in MS isolated triple mutant embryos (n = 4). (F) In triple mutant embryos in which the E blastomere was isolated, the *nhr-25*::YFP marker was detected (n = 5).

To determine the origin of the extra MS-specific tissues being made in *end(-); pop-1(-)* embryos, laser ablation studies were done in embryos expressing the *cup-4*::GFP, *myo-2*::GFP, and *unc-120*::GFP markers. As it was previously noted, *end-3(ok1448); pop-1(RNAi)* embryos made an average of 6.1 ± 0.2 coelomocytes (n = 77) and *end-1,3(ok1448); pop-1(RNAi)* embryos made an average of 9.3 ± 0.2 coelomocytes (n = 80). As a control, MS, E or both blastomeres were ablated in *end-3(ok1448)* or *end-1,3(ok1448)* embryos (Table 3). In the MS ablated *end-3(ok1448)* embryos no coelomocytes were detected (n = 5), while 1/5 MS ablated *end-1,3(ok1448)* embryos contained a weak expression of *cup-4*::GFP (0.2 ± 0.2, n = 5), most likely due to an incomplete ablation. Ablations of the E blastomere in these embryos, resulted in the expected expression of *cup-4*::GFP, in which *end-3(ok1448)* made 4.0 ± 0.0 coelomocytes (n = 2) and *end-1,3(ok1448)* made 3.6 ± 0.2 coelomocytes (n = 5) (Table 3). When MS and E were both ablated in *end-1,3(ok1448)* mutants, none of the embryos expressed *cup-4*::GFP (0.0 ± 0.0, n = 3). Contrary to the control experiments, all MS ablated embryos in *end-3(ok1448); pop-1(RNAi)* and *end-1,3(ok1448); pop-1(RNAi)* expressed *cup-4*::GFP (3.0 ± 0.6, n = 4; 6.7 ± 0.5, n = 3, respectively) (Fig. 21D; Table 3), suggesting that another blastomere is making coelomocytes. In addition to MS ablations, the E cell was ablated in these embryos. As expected the MS blastomere in E ablated *end-3(ok1448); pop-1(RNAi)* embryos expressed *cup-4*::GFP (3.0 ± 0.5, n = 5), as well as E ablated *end-1,3(ok1448); pop-1(RNAi)* embryos which produced an average of 4.7 ± 0.7 coelomocytes (n = 3) (Fig. 21C; Table 3). Thus far, the data suggests that both MS and E produce coelomocytes in the absence of *end* genes and *pop-1*. Therefore, MS

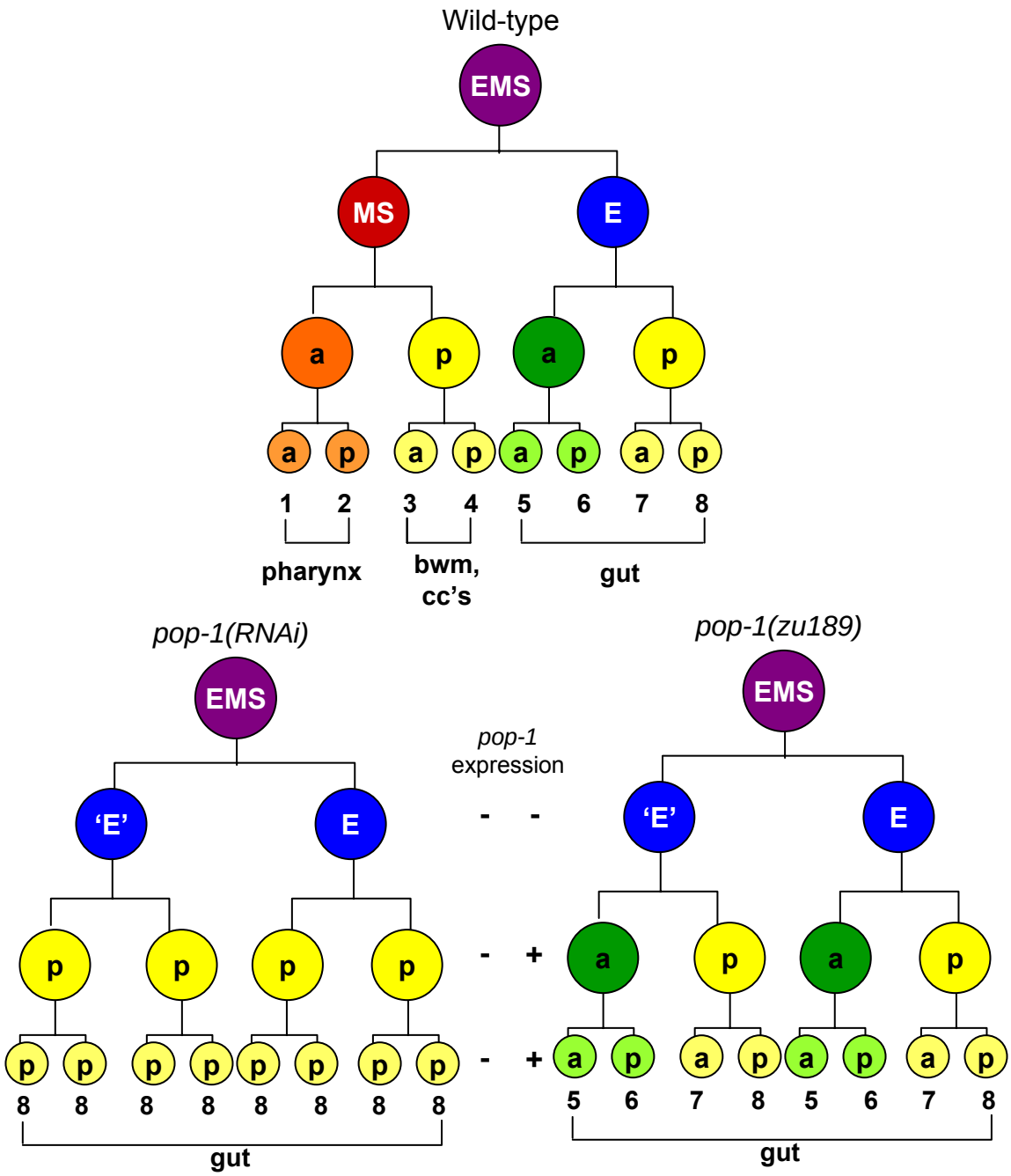
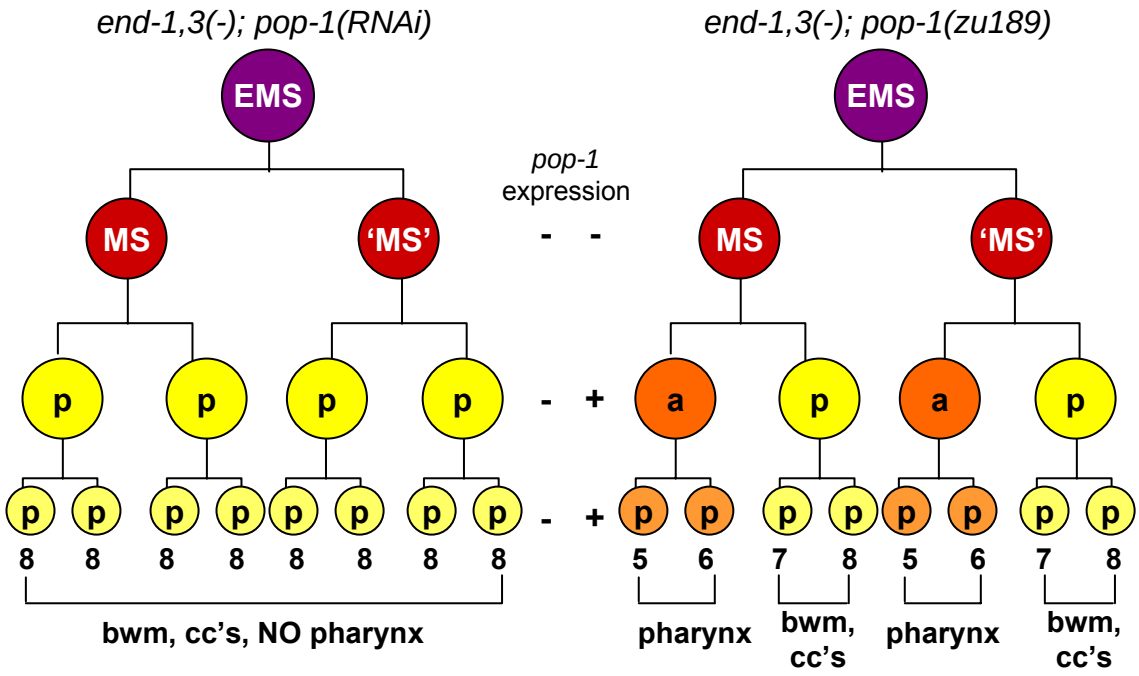


Fig. 22. Schematic diagram of the anterior/posterior patterning observed in wild-type, *pop-1(RNAi)*, *pop-1(zu189),* and *end-1,3; pop-1(RNAi)*, and *end-1,3; pop-1(zu189)* embryos. Wild-type embryos display a normal anterior/posterior cell division resulting in specification of different tissues. The anterior daughter cell of MS gives rise to pharynx, posterior MS daughter cell gives rise to body wall muscle (bwm) and coelomocytes (cc's), while the anterior and posterior daughter cells of E give rise to gut. This pattern is altered in *pop-1(RNAi)* pop-1(zu189), and *end-1,3; pop-1(RNAi)* mutant embryos. Figure composited by myself and M. Maduro.

Fig. 22 continued



and E ablations were conducted and no coelomocytes were made in *end-1,3(ok1448); pop-1(RNAi)* mutants (0.0 ± 0.0, n = 4), except for 1/4 partial embryos expressing weak *cup-4*::GFP in the *end-3(ok1448); pop-1(RNAi)* mutants, again most likely due to incomplete ablations. To further validate these findings, MS or E were isolated in *end-1,3(ok1448; pop-1(RNAi)* embryos using a laser microbeam to ablate all other cells. The MS isolated embryos produced an average of 5.0 ± 0.4 coelomocytes (n = 4) and E isolated embryos produced 5.5 ± 0.3 coelomocytes (n = 6) (Table 3). As expected in control *end-1,3(ok1448)* embryos coelomocytes were made in all MS isolated embryos (4.0 ± 0.0, n = 5) and none made in E isolated embryos (0.0 ± 0.0, n = 4) (Table 3). Taken together, these results reveal that coelomocytes and GLP-1-independent pharynx are made by both MS and E in *end; pop-1* embryos. This implies that POP-1 is not essential for production of mesoderm tissue from MS, in addition, POP-1 and/or END-1,3 may repress MS fate in the E cell.

Restored MS-like fates in end-1,3; pop-1 mutants require normal pathways for MS specification

It has been shown that *med-1,2*, as well as *tbx-35* and *ceh-51* together, are vital for production of coelomocytes and GLP-1-independent pharynx (Broitman-Maduro et al., 2009; Maduro et al., 2001). In addition, expression of *tbx-35* and *ceh-51* continues to occur in MS in *pop-1(-)* embryos (Broitman-Maduro et al., 2009). Therefore, the extra coelomocytes and pharynx cells that are detected in *end; pop-1* mutants may be developing through activation of the same genes that specify MS in wild-type embryos.

To confirm this, *cup-4*::GFP expression was observed in *med-1(ok804); med-2(cx9744); end-1(ok558) end-3(ok1448)* (*med-1,2; end-1,3*) embryos, as well as in *med-1(ok804); med-2(cx9744);end-1(ok558) end-3(ok1448); pop-1(RNAi)* (*med-1,2; end-1,3; pop-1*) embryos. As expected, the *med-1,2; end-1,3* mutant strain did not express coelomocytes (0.0 ± 0.0, n = 52) and *med-1,2; pop-1* mutant embryos produced 1.7 ± 0.2 coelomocytes (n = 60) (Table 3). Production of coelomocytes dramatically decreased in *med-1,2; end-1,3; pop-1(RNAi)* embryos to 0.1 ± 0.2 (n = 57) compared to *end-1,3(ok1448); pop-1(RNAi)* embryos (10.7 ± 0.3, n = 77, P<0.001) (Table 3; Fig. 19F,G). Preliminary data also suggested that *tbx-35* may have a role in production of coelomocytes in *end-3(ok1448); pop-1(RNAi)* embryos, since *tbx-35(-); end-3(ok1448); pop-1(RNAi)* embryos produced an average of 3.8 ± 0.5 coelomocytes (n = 17, P<0.001 compared to *end-1,3(ok1448); pop-1(RNAi)* embryos). To investigate this further, *cup-4*::GFP expression was analyzed in *tbx-35; end-1,3 ceh-51; pop-1(RNAi)* mutant embryos. As expected, coelomocytes were significantly reduced in *tbx-35(-); end-1,3(-) ceh-51(-); pop-1(RNAi)* embryos, to 0.1 ± 0.1 (n= 35) compared to *end-1,3; pop-1* mutant embryos (P<0.001) (Table 3; Fig. 19F, H). Also as control experiments, expression of *cup-4*::GFP in *tbx-35; end-1,3 ceh-51* mutant embryos was hardly detected (0.1 ± 0.0, n = 122) and was not detected in *tbx-35; ceh-51; pop-1(RNAi)* embryos (0.0 ± 0.0, n =64).

A similar pattern was observed in mutants carrying the *pha-4*::GFP marker. Quadruple mutant embryos, *med-1,2; end-1,3* and *tbx-35; ceh-51, end-1,3*, made 29.9 ± 1.1 (n = 12) and 30.3 ± 1.0 (n = 21) pharynx cells, respectively (Fig. 20K, M), while addition of *pop-1(RNAi)* produced even fewer pharynx cells in *med-1,2; end-1,3; pop-*

1(RNAi) (22.4 ± 0.9, n = 24) and *tbx-35; end-1,3 ceh-51; pop-1(RNAi)* embryos (22.1 ± 1.4, n = 19) (Fig. 20L, N), compared to the ~30 cells made without *pop-1(RNAi)* in the quadruple mutant embryos and *pop-1(RNAi)* alone (21.3 ± 0.8, n = 18) (Owraghi et al., 2009). These results demonstrate that the MS-derived tissues made in *end-1,3; pop-1* embryos require the same genes involved in normal MS specification and the fact that more than 8 coelomocytes are produced (as would be expected from two MS-like cells in these mutant embryos) further suggests that POP-1 plays a role in MS lineage asymmetries that give rise to coelomocytes, which has recently been reported to occur in post-embryonically-specified coelomocytes (Amin et al., 2009).

The production of pharynx cells and coelomocytes from E in *end-1,3; pop-1(RNAi)* embryos suggests that POP-1 contributes to repression of MS fate in E. As previously mentioned, *tbx-35* was not detected in *pop-1* mutant embryos in the E lineage, either by in situ hybridization or a *tbx-35*::GFP reporter with 605 bp of putative *tbx-35* promoter (Broitman-Maduro et al., 2006). However, ectopic expression of *tbx-35* in the E lineage was detected with a 734 bp promoter (Fig. 23A, B) [Shetty, P., Lin, R., personal communication; results reproduced with their reporter (Owraghi et al., 2009)]. In addition, it was recently reported that endogenous *ceh-51* (Fig. 23C, D) and *ceh-51*::GFP reporter is often detected in the early E lineage of *pop-1(RNAi)* embryos (Broitman-Maduro et al., 2009). These findings, in addition to the consistent observation that the E cell produces MS-derived tissues in *end-1,3; pop-1* mutant embryos, suggests that loss of *end-1,3* may synergize with loss of *pop-1* in activation of MS genes. In fact, low levels of *ceh-51* transcripts were detected in *end-1,3(ok1448)* mutants (Fig. 23E)

(Owraghi et al., 2009). Moreover, strong expression of *ceh-51* in both E and MS lineages was detected in *end-3; pop-1(RNAi)* embryos (Fig. 23F) (Owraghi et al., 2009). These results confirm that the MS tissues made by E in *end; pop-1* embryos correlate with ectopic activation of MS specification in E, and also provide evidence that POP-1 and END-1,3 contribute to the repression of early MS genes in the E lineage.

Loss of TCF/LEF corepressor, UNC-37/Groucho, and histone deacetylase, HDA-1, results in ectopic coelomocytes

Studies in *Drosophila* and mammals have shown that the ability of TCF/LEF proteins to activate or repress transcription is determined by the Wnt signaling pathway, which controls the recruitment of coactivators and corepressors (Barker et al., 2000). In the absence of Wnt activity, TCF/LEF proteins control transcriptional repression through the recruitment of a corepressor, Groucho (Barker et al., 2000; Chen and Courey, 2000; Fisher and Caudy, 1998; Roose et al., 1998). It has also been shown, through genetic and biochemical studies, that Groucho and histone deacetylase interact during *Drosophila* embryogenesis (Chen et al., 1999). Each of these factors, however, does not bind DNA directly and is brought to promoters by interactions with sequence-specific DNA-binding transcription factors. In *C. elegans*, the Groucho homolog, UNC-37 has been shown to play a role in post-embryonic neuronal cell fate determination (Pflugrad et al., 1997). In addition, UNC-37 and the histone deacetylase, HDA-1, have been shown to be recruited to POP-1, forming a complex to repress transcription of *end-1* in the MS lineage (Calvo et al., 2001). In the absence of *unc-37*, *end-1*::GFP was somewhat reduced when

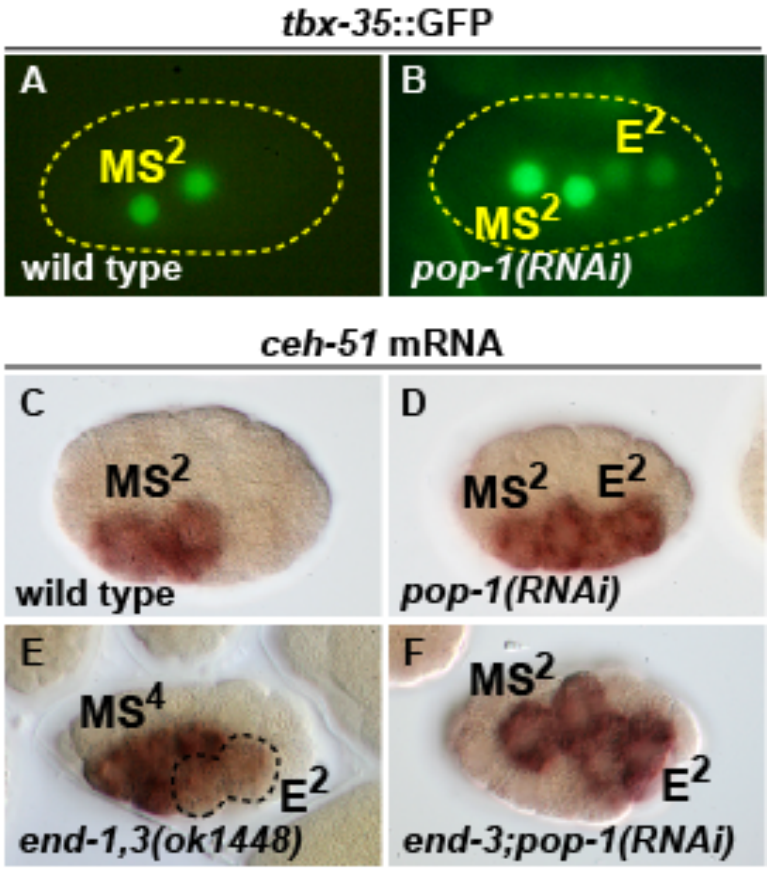


Fig. 23. Expression of MS specification factors in *pop-1(RNAi)* and *end-1,3* mutant backgrounds. (A, B) Expression of the *tbx-35*::GFP reporter in wild-type and *pop-1(RNAi)* embryos. One hundred percent (n = 40) of expressing embryos showed MS lineage-only expression in wild-type, while twelve percent (n = 67) of *tbx-35*::GFP-expressing *pop-1(RNAi)* embryos showed ectopic expression in the E lineage as shown; seventy-five percent of embryos overall had weak E lineage expression. (C-F) In situ hybridization (performed by G. Broitman-Maduro) with a *ceh-51* probe. (C) Wild-type staining pattern observed in ninety-one percent of embryos in the early MS lineage (MS^2 as shown, or MS^4; n = 101). (D) Ectopic expression of *ceh-51* is observed in the early E lineage in eighty-six percent (n = 44) of embryos, while the remaining embryos showed either normal or no staining (C and D also reported in Broitman-Maduro et al., 2009). (E) Weak ectopic expression of *ceh-51* in the E lineage (in E^2 as shown or at E^4) observed in forty-one percent (n = 209) of *end-1,3(ok1448)* embryos. (F) Expression of *ceh-51* in MS and E was seen in eighty-eight percent (n = 45) of *end-1,3(ok1448); pop-1(RNAi)* embryos. The remaining embryos were either unstained or appeared normal. Figure composited by M. Maduro from data acquired by M. Maduro and G. Broitman-Maduro. Legend modified from Broitman-Maduro et al., 2009.

compared to wild-type embryos, however ~63% of *hda-1(RNAi)* embryos expressed *end-1*::GFP in the MS and E lineages (Calvo et al., 2001). When *hda-1* and *unc-37* were both inhibited, via RNAi, nearly all embryos expressed *end-1*::GFP in MS and E lineages (~95%, n = 36), suggesting that HDA-1 and UNC-37 work together to repress *end-1*::GFP in MS descendants, although de-repression of *end-1* was found in other non-MS descendants (i.e., AB lineage), as well (Calvo et al., 2001).

Although this study revealed a mechanism of how POP-1 functions to repress *end-1* in the MS lineage, it became of interest to determine whether depletion of *hda-1*, *unc-37*, and *end-1,3* together result in ectopic MS-like tissues, similarly found in *pop-1; end-1,3* mutant embryos. Since UNC-37 and HDA-1 function together with POP-1 to repress endoderm genes in the MS blastomere, I wanted to determine if depletion of these factors will result in the same phenotype as *end(-); pop-1(-)* embryos. If the *end-1,3(-); unc-37(RNAi); hda-1(RNAi)* embryos produce extra MS-specific tissues, like *end(-); pop-1(-)* embryos, we can conclude that UNC-37 and HDA-1 also function with POP-1 to repress MS specification in the E cell. However, the results may not be as obvious as *end(-); pop-1(-)* embryos, since corepressors and histone deacetylase factors can have more than one regulatory function within other lineages of the developing embryo.

Since coelomocytes are only made by the MS lineage, the *cup-4*::GFP reporter was used for preliminary observations. Embryos expressing *cup-4*::GFP were scored in *end-1,3(ok1448); unc-37(RNAi)*, in *end-1,3(ok1448); hda-1(RNAi)*, and in *end-1,3; hda-1(RNAi); unc-37(RNAi)* mutant backgrounds. In *end-1,3(ok1448); unc-37(RNAi)* embryos, an average of 6.0 ± 0.2 (n = 55) coelomocytes were made, while embryos

rescued for *end-1,3* [*end-1,3(+); unc-37(RNAi)*] produced an average of 4.5 ± 0.1 (n = 64) coelomocytes. On the other hand, *end-1,3(ok1448); hda-1(RNAi)* embryos produced more coelomocytes compared to *end-1,3; unc-37* mutant embryos (8.2 ± 0.3, n = 25). However, *end-1,3(+); hda-1(RNAi)* embryos continued to produce the normal number of embryonic coelomocytes (4.29 ± 0.3, n = 7). When both, *hda-1* and *unc-37* were absent in the *end-1,3* mutant background, embryos produced an average of 7.4 ± 0.3 coelomocytes (n = 34), while *end-1,3(+); hda-1(RNAi); unc-37(RNAi)* embryos made 5.5 ± 0.5 (n = 17) coelomocytes.

As a control experiment, *cup-4*::GFP expression was analyzed in *hda-1(RNAi)* embryos alone, as well as in *unc-37(RNAi)* embryos and *hda-1(RNAi); unc-37(RNAi)* embryos, and were compared to wild-type embryos. The *hda-1(RNAi)* embryos produced a significant increase in the number of coelomocytes (7.7 ± 0.2, n = 81) coelomocytes compared to wild-type embryos (3.7 ± 0.2, n = 105, $P<10^{-34}$), while *unc-37(RNAi)* embryos produced slightly more coelomocytes (4.8 ± 0.1, n = 82) compared to wild-type embryos ($P<10^{-6}$). In addition, *unc-37(RNAi); hda-1(RNAi)* embryos produced 3.7 ± 0.1 coelomocytes (n =100), which is about the same as wild-type embryos (P=1.0). It was interesting to find that *hda-1(RNAi)* embryos produce significantly more coelomocytes than wild-type embryos, but there was not a very significant difference between *hda-1(RNAi)* embryos and *end-1,3(ok1448); hda-1(RNAi)* embryos (8.2 ± 0.3, n = 25, P=0.1). The data suggests that the extra coelomocytes found in *end-1,3(ok1448); hda-1(RNAi)* embryos are not *end-1,3* dependent. On the other hand, in the absence of *end-1,3*, *unc-37(RNAi)* embryos produced more coelomocytes (6.0 ± 0.2, n = 55) when compared to

unc-37(RNAi) embryos alone (4.8 ± 0.1, n = 82, $P<10^{-8}$), suggesting that the extra coelomocytes may be dependent on the loss of *end-1,3* similarly found in *end-1,3(ok1448); pop-1(RNAi)* embryos. Furthermore, *end-1,3(ok1448); unc-37(RNAi); hda-1(RNAi)* embryos produced significantly more coelomocytes (7.4 ± 0.3, n = 34), as well, compared to *unc-37(RNAi); hda-1(RNAi)* embryos (3.7 ± 0.1, n = 100, $P<10^{-14}$). The data reveal that extra coelomocytes are produced and may be a result of E adopting an MS-like fate in *end-1,3; hda-1(RNAi); unc-37(RNAi)* embryos, similar to *end-1,3(ok1448); pop-1(RNAi)* embryos.

Laser ablation studies were conducted to elucidate the source of the extra coelomocytes produced in *end-1,3; hda-1(RNAi); unc-37(RNAi)* embryos. In these experiments, the MS blastomere was ablated in each mutant strain. In *end-1,3(ok1448); hda-1(RNAi)* embryos approximately 2.0 ± 0.3 (n = 3) coelomocytes were produced, on average, and when MS and E blastomeres were both killed in these embryos, no coelomocytes were made (n = 5), suggesting that E may be making extra coelomocytes, and possibly that MS is also making an average of two more coelomocytes than normal since *end-1,3(ok1448); hda-1(RNAi)* embryos produce about 8.2 ± 0.3 (n =25) coelomocytes. To determine this, the E blastomere must also be ablated and the number of coelomocytes made by MS must be counted. Although the extra coelomocytes made in *end-1,3(ok1448); hda-1(RNAi)* embryos are not due to the absence of *end-1,3*, it may be that the absence of *hda-1* activity that results in E to make MS-derived coelomocytes, as well as MS possibly making more coelomocytes. On the contrary, some preliminary laser ablation data showed that when the MS blastomere was ablated in *end-1,3(ok1448);*

unc-37(RNAi) embryos an average of 3.0 ± 0.0 (n = 2) coelomocytes were produced. Although it is difficult to make solid conclusions based on a small sample size, the findings do suggest the possibility that the E blastomere is making extra coelomocytes and may, in fact, be *end-1,3* dependent since *end-1,3(ok1448); unc-37(RNAi)* embryos produced an average of 6.0 ± 0.2 (n =55) coelomocytes. Further MS ablations, as well as MS and E ablations, would be necessary, however, to confirm this possibility. Similarly, more MS ablations need to be done in *end-1,3(ok1448); hda-1(RNAi); unc-37(RNAi)* since the preliminary findings included a sample size of 2. In these MS-ablated embryos no coelomocytes were observed, suggesting that the extra coelomocytes made in *end-1,3; hda-1(RNAi); unc-37(RNAi)* embryos may be originating from the MS cell.

The reasoning behind why the MS blastomere is producing more coelomocytes in the absence of these genes may be due to a required role for UNC-37 and HDA-1 in later asymmetric divisions that require POP-1 within the MS cell. Since both *hda-1* and *unc-37* work together to ensure maximum repression of inappropriate cell fate determination (Calvo et al., 2001), loss of these genes, particularly *hda-1*, may result in cells of the MS lineage, that are destined to give rise to a specific tissue-type transform into coelomocytes. This, in fact, has been observed before, post-embryonically however, in which the absence of *lin-12* (a transmembrane Notch-signaling receptor) the sex myoblasts, which are derived from the post-embryonic M blast cell, transform into coelomocytes (Greenwald et al., 1983; Harfe et al., 1998). It was found that two extra coelomocytes were present in null *lin-12* mutant embryos, while vulval muscles were missing. In addition to these findings, *hda-1* has been shown to be required for proper

gonadogenesis and vulval development and linked to the Notch signaling pathway, since *lag-2* (a Notch ligand) has been found to be its target gene (Dufourcq et al., 2002). Therefore, it is possible that during embryonic development, *hda-1* is required for proper specification of a particular cell type in the MS cell, and if *hda-1* is not present improper cell fate transformations may result, in this case the resultant embryo giving rise to extra coelomocytes.

The E blastomere produces epidermal tissue and MS tissues in end; pop-1 embryos

As previously described, loss of *pop-1* results in MS adopting an E-like fate and loss of *end-1* and *end-3* results in E adopting a C-like fate, indicating the possibility that in *end-1,3; pop-1* triple mutant embryos MS and/or E could make cells that are normally derived from the C blastomere. In addition, the Caudal-like regulator PAL-1, which specifies the fate of C an D blastomeres, is present in the early MS and E lineages, and is the reason for the C transformations that result in mutations that occur in the SKN-1/MED-1,2 pathway (Broitman-Maduro et al., 2006; Broitman-Maduro et al., 2009; Hunter and Kenyon, 1996; Maduro et al., 2005a). To assess this possibility, the *end-1,3; pop-1* mutant strain was constructed to carry an epidermal marker, *nhr-25*::YFP (Baugh et al., 2005a). A laser microbeam was used to ablate all the cells except for MS or E in mutant embryos expressing the *nhr-25*::YFP marker and resultant partial embryos were evaluated. Since *nhr-25*::YFP is a cytoplasmic marker, epidermal cells could not be counted, therefore only expression of the marker in the isolated cells were noted. To ensure that the expression of the marker was specific to C-type cells, the C blastomere

was isolated in wild-type embryos and were found to express *nhr-25*::YFP (n = 5). MS-isolated wild-type embryos, on the other hand, did not express *nhr-25*::YFP (n = 3) (Table 3). Since MS and E both adopt a C-like fate in *skn-1* mutant embryos, expression of the marker in such resultant partial embryos were analyzed, as well, and both MS- and E-isolated *skn-1(RNAi)* embryos expressed the *nhr-25*::YFP marker (n = 3; n = 9, respectively). Gut granules were also not detected in E-isolated *skn-1(RNAi)* embryos, confirming that a complete E-to-C-like transformation took place. In addition to these controls, isolations were done in *pop-1(RNAi)* or *end-1,3(ok1448)* embryos. MS isolations in *pop-1(RNAi)* embryos resulted in no expression of *nhr-25*::YFP (n = 3), as well as no expression in E-isolated mutant embryos (n = 4), while E-isolated *end-1,3(ok1448)* embryos showed expression of the marker (n = 6) (Table 3). When MS was isolated in *end-1,3; pop-1(RNAi)* embryos, *nhr-25*::YFP expression was not detected (n = 4) (Fig. 21E; Table 3). However, in triple mutant embryos, which the E blastomere was isolated, *nhr-25*::YFP was expressed (n = 5) (Fig. 21F; Table 3). The fact that E-isolated *end-1,3(ok1448); pop-1(RNAi)* embryos express the epidermal marker, *nhr-25*::YFP, suggests that descendants of E may adopt either MS- or C-like fates.

Preliminary results also suggested that the E blastomere may not be adopting the MS fate completely in *end-1,3; pop-1* mutant embryos. Another MS-specific reporter was used to mark body muscle using *unc-120*::GFP and isolations were performed in mutant embryos expressing this marker, as well. As previously described, body muscle is made by the MS cell, by C and D blastomeres, as well as one muscle cell made by AB. Therefore, isolating the cells in the embryo provided a more specific readout as to

whether MS or E expressed *unc-120*::GFP in the mutant backgrounds. When E was isolated in wild-type embryos, *unc-120*::GFP expression was not detected (0.0 ± 0.0, n = 4). However, E isolated *end-1,3(ok1448)* embryos contained an average of 15.3 ± 0.7 body muscle cells (n = 3), consistent with the fact that E adopts a C-like fate in the absence of the *end* genes. In addition, MS isolated *end-1,3(ok1448)* embryos produced an average of 18.7 ± 2.0 cells, which is very close to the known 23 body muscle cells made by the MS blastomere (Sulston et al., 1983). However, when the E cell was isolated in *end-1,3(ok1448); pop-1(RNAi)* embryos, an average of 10.2 ± 1.2 cells were made (n = 6) (Table 3). The data reveals that the number of body muscle cells produced in the E blastomere of mutant embryos reduce compared to the cells produced by the E blastomere of *end-1,3(-)* embryos. In addition, these E-isolated mutant embryos did not produce gut, suggesting the likelihood that the E cell may be producing a different type of tissue from another lineage. Another possible explanation for the reduced number of body muscle cells found in the E-isolated mutant embryos could be a result of cells from the E lineage undergoing apoptosis. However, the latter is likely not the case since preliminary evidence shows that the E lineage also makes epidermal-like cells. Therefore, the next step was to investigate whether or not E is making both MS- and C-like tissues simultaneously. Laser ablation studies were conducted in the triple mutant strain which was constructed to carry both the *cup-4*::GFP and the *nhr-25*::YFP markers (Baugh et al., 2005a; Patton et al., 2005); the two reporters were distinguished with appropriate filter sets (Miller et al., 1999). MS blastomeres isolated in *end-1,3(ok1448); pop-1(RNAi)* embryos generated coelomocytes (4.0 ± 0.0, n = 4) but not epidermal cells

(n = 4), while E-isolated embryos made coelomocytes all the time (5.5 ± 0.3, n = 6) and, simultaneously, epidermal cells part of the time (4/6 embryos) (Table 3). This led to the conclusion that in *end; pop-1* mutant embryos, E, but not MS, adopts a mixed fate that includes production of MS- and C-derived tissues.

Discussion

The data presented in this chapter draws attention to how the blastomere specification pathways in early *C. elegans* embryo can undergo phenotype prevalence. Specification of MS is the result of activation of *tbx-35/ceh-51* and specification of E results due to activation of *end-1* and *end-3* (Broitman-Maduro et al., 2006; Broitman-Maduro et al., 2009; Maduro et al., 2005a). Once a regulator involved in this specification pathway is removed, factors that are normally not activated in a cell become endogenously active in that cell, but fail to function due to a dominant factor that prevails instead. For example, in *pop-1* mutant embryos *end-1* and *end-3* become activated in MS, but *tbx-35* and *ceh-51* continue to be activated in MS as well. In addition, maternal PAL-1 protein, which specifies the C lineage, is present in the early MS and E lineages (Hunter and Kenyon, 1996). Collectively, it is found that in *pop-1* mutant embryos all the factors that specify E, MS and C are present in these blastomeres all at the same time. Why, then, does MS adopt an E-like fate in *pop-1* mutant embryos if all of these specifying factors are simultaneously active? The answer, simply, relies on the fact that, *end-1* and *end-3* remain to dominate in function over the other specification factors, *tbx-35*, *ceh-51* and *pal-1*, through some unknown mechanism besides the fact that the

Wnt/MAPK signaling pathways influence the decision of posterior daughter of EMS to specify the E fate. However, if the *end* genes are eliminated in addition to *pop-1*, then the E cell transforms into an MS-like cell, suggesting that TBX-35 and CEH-51 take dominance, next, over PAL-1, just as it does in the normal MS cell. But, unlike the strict transformation of the MS cell that occurs in different mutant backgrounds (*pop-1(RNAi)*, *med-1,2(-)*, *tbx-35(-); ceh-51(-)*, etc.), the E blastomere adopts a mixed fate, in which both MS- and C-like tissues are made due to the presence of TBX-35, CEH-51 and PAL-1 in the E blastomere of *end-1,3; pop-1* mutant embryos [Figure 24; (Owraghi et al., 2009)]. Evidence of E adopting mixed fates has been demonstrated in prior studies, as well. In *end-3* mutants, a small percentage of embryos develop "half-guts" in which only half of the intestine was present (Maduro et al., 2005a). In addition, a 4-D recording of one lineage of an *end-3(zu247)* mutant embryo showed that the E grand-daughter followed a lineage pattern similar to an MS grand-daughter (Maduro et al., 2005a).

As described earlier, expression of *tbx-35* in the E lineage was found to be weaker than in the MS lineage of *pop-1(RNAi)* embryos (Broitman-Maduro et al., 2009). However, expression of *ceh-51* is present at similar levels in both the MS and E lineages in most *pop-1(RNAi)* embryos (Broitman-Maduro et al., 2009; Owraghi et al., 2009), but is no longer detected in *tbx-35; pop-1(RNAi)* mutant embryos, suggesting that the *ceh-51* expression is dependent on *tbx-35* and *pop-1* (Broitman-Maduro et al., 2009). In addition, expression of PAL-1 is present at equal levels in MS and E lineages of wild-type embryos and has been found to be required for production of gut in conjunction with

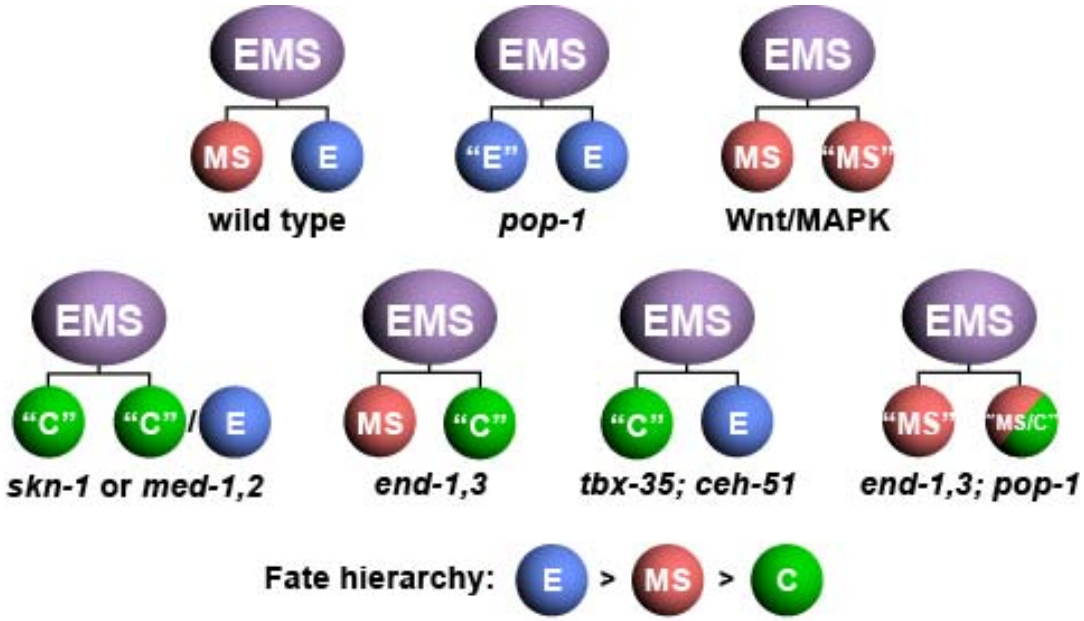


Fig. 24. Summary of fate hierarchies revealed by EMS daughter cell fate transformations in various mutant backgrounds. Loss of *pop-1* results in an MS to E transformation (Lin et al., 1995); loss of Wnt/MAPK function causes an E to MS transformation (Thorpe et al., 1997); for *skn-1* or *med-1,2* mutant embryos MS adopts a C-like fate, while E appears to adopt either an E or C fate (Bowerman et al., 1992; Maduro et al., 2001); loss of *end-1,3* results in an E to C transformation (Maduro et al., 2005a; Zhu et al., 1997); loss of *tbx-35* and *ceh-51* results in a MS to C transformation (Broitman-Maduro et al., 2009); and loss of *end-1,3* and *pop-1* together results in a restored partial MS lineage, and an apparent mixed fate from E that is a combination of MS and C fates (Owraghi et al., 2009). Figure composited by M. Maduro. Legend modified from Owraghi et al., 2009.

SKN-1 and POP-1 (Hunter and Kenyon, 1996; Maduro et al., 2005b). Therefore, the fact that E adopts a mixed, MS/C-like fate is consistent with the findings that the MS and C specification factors are still expressed and active in these triple mutant embryos. Taken together, the findings reveal a hierarchy of E>MS>C fates, in which the EMS daughters adopt an E-like fate first revealed in the absence of *pop-1*; in the absence of *end-1,3* and *pop-1* EMS divides into two MS-like daughters, while E adopts both MS- and C-like fates; and finally in the absence of *end-1,3*, *pop-1*, *tbx-35* and *ceh-51* (or *med-1* and *med-2*), it is very likely that the EMS daughters adopt a C-like fate, in which PAL-1 is the regulator responsible for the transformation that occurs. But to make this conclusion, all the cells except for MS or E need to be ablated in these mutant embryos expressing *nhr-25*::YFP.

The combinatorial regulation of cell fate specification observed in *C. elegans* has been found to be a common theme in other organisms, as well. In the *Drosophila* ventral nerve cord, for example, it has been found that the HLH gene, *collier*, functions to restrict neuronal identity in several different ways in the early lineage. However, as a final regulatory step, *collier* is required for making two overlapping combinatorial factors different in order to give rise to a specific, desired neuronal fate (Baumgardt et al., 2007). Furthermore, in mouse lymphocytic cells a distinct set of transcription factors have been found to be responsible for specification of the B cell fate (cells that are responsible to make antibodies against antigens found in the infected organism) (Medina et al., 2004). The group of researchers report that a set of transcription factors, PU.1, Ikaros, E2A, EBF, and Pax5 function in early B cell development (Medina et al., 2004). Based on the

observations made in the absence of these various genes within the mouse lymphocytic B cells, the group has elucidated a hierarchical regulatory network for specification and commitment to the B cell fate (Medina et al., 2004). Collectively, it is evident that multiple pathways are required for proper specification of a desired cell fate within various organisms.

Chapter 5: Conclusions

The NK-2 class homeodomain factor, CEH-51, and the T-box factor, TBX-35, have overlapping function in C. elegans mesoderm development

The findings reported above reveal new information about the endomesoderm gene regulatory network in the *C. elegans* early embryo, however there are still unknown factors and regulators that have yet to be discovered. Prior to beginning my projects, the newly identified role of TBX-35 in MS-specification had just been published (Broitman-Maduro et al., 2006). It was reported that TBX-35 is required for the majority of MS fates to be made, as well as sufficient for specification of early MS-derived tissues (i.e., posterior pharynx and body wall muscle). The MS lineage, however, is reported to give rise to other MS-specific tissues, as well (i.e., coelomocytes and somatic gonad precursor cells, Z1 and Z4) (Sulston et al., 1983). Coelomocytes, being specified approximately 30-40 minutes later after the pharynx primordium is made, was the next set of MS-derived tissues that was of interest to determine if TBX-35 was required for its specification, as well. The *cup-4*::GFP marker was obtained upon request from a different laboratory (Patton et al., 2005) and was used to detect coelomocytes in *C. elegans* embryos. The coelomocyte marker was first used to test and determine if it was expressed in the right number of cells (expected to observe four embryonically-derived coelomocytes) and expressed in the right cell, which was the MS cell. The *cup-4*::GFP marker was in fact found to be MS-specific but the next question was whether or not TBX-35 is necessary and sufficient for coelomocyte specification. The expression of the *cup-4*::GFP marker was still observed in the absence of *tbx-35*, however ectopic

expression of the marker was detected when *tbx-35* was over-expressed via a heat-shock promoter. These results suggested that *tbx-35* is sufficient but not necessary for coelomocyte specification.

The findings also provided further evidence that another MS-specific factor must be involved, in parallel with *tbx-35*, to specify for these later MS-derived tissues. Preliminarily, some of the *tbx-35* null mutant embryos would arrest before hatching, while others would elongate and sometimes hatch and then arrest at the larvae stage when grown at 20ºC (Broitman-Maduro et al., 2006). However when embryos were grown at lower temperatures (15ºC), the majority of the embryos displayed a phenotype more similar to wild-type embryos, in which they would hatch, arrest as larvae, and most of them appearing to have a normal amount of pharynx cells (Broitman-Maduro et al., 2009). The *tbx-35* mutant embryos seem to be able to compensate for the loss of *tbx-35* better at lower temperatures than at higher temperatures. It was this initial observation that suggested the possibility of another factor involved in proper specification of MS tissues. With several different attempts to identify the other factor involved in specification of MS tissues, it was until recently when the new regulator, *ceh-51*, had been identified to be required for proper development of MS tissues. It was found that in the absence of both *tbx-35* and *ceh-51*, embryos display similar abnormalities as *med-1,2* mutant embryos (Broitman-Maduro et al., 2009). It is reported that *ceh-51; tbx-35* double mutants displayed a strong embryonic arrest that is not temperature sensitive and is comparable to that of *med-1,2* double mutants grown at both 20ºC and 15ºC (Broitman-Maduro et al., 2009). Also, development of MS-derived pharynx was abnormal when

grown at both temperatures, in which embryos only displayed a partial grinder. In addition the number of pharynx cells was reported to be lower in these double mutant embryos and similar to the low number of pharynx cells found in *med-1,2* double mutants (Broitman-Maduro et al., 2009). Finally, no coelomocytes were produced in *tbx-35; ceh-51* mutant embryos, comparable to that of *med-1,2* double mutant embryos (0.19 ± 0.04, n = 124 and 0.07 ± 0.03, n = 34, respectively). In addition, with the use of a hypodermal marker, *nhr-25*::YFP, we were able to detect that *tbx-35; ceh-51* double mutants undergo an MS-to-C-like transformation, just as it is found in *med-1,2* double mutant embryos.

Collectively, the results reveal that *tbx-35* and *ceh-51* are both required for proper development of MS-derived tissues. However, a question still remains as to what other factor is responsible for activation of *ceh-51* besides *tbx-35*, since *ceh-51* continues to be expressed, although weakly, in the MS and E blastomeres in *tbx-35* mutant embryos. Although, *tbx-35; pop-1(RNAi)* embryos fail to express *ceh-51*, suggesting that *ceh-51* expression is dependent on both *tbx-35* and *pop-1*. Another possibility is that loss of *pop-1* causes *end-1,3* to be activated through the SKN-1 pathway, as well as through positive regulation from PAL-1, and results in further repression of *ceh-51*. Therefore, future work is aimed at elucidating the presence and function of other MS-specific factors and factors that are activated downstream of TBX-35 and CEH-51 in order to uncover further conserved aspects of mesoderm development in *C. elegans*.

Roles of Wnt effector POP-1/TCF in the C. elegans endomesoderm specification gene network

As a separate project, it was of interest to investigate the possibility of additional roles of POP-1, which was suggested by other results. It was proposed that POP-1 does not only act as an activator of endoderm genes in the E blastomere, but is also involved in MS specification (Lin et al., 1998; Lin et al., 1995). In addition, our lab had recently discovered that POP-1 acts positively, with SKN-1, to specify the MS blastomere in the related nematode *C. briggsae* (Lin et al., 2009). Therefore, experiments were designed to investigate whether POP-1 has such a role in the MS cell in *C. elegans*. Since the absence of *pop-1* results in an MS-to-E-like transformation, it was necessary to deplete the endoderm specifying genes, *end-1* and *end-3* to determine if *pop-1* has any additional roles.

Initial studies began with the use of a strain that contained a deletion of the DNA-binding domain of the *end-3* gene (Maduro et al., 2005a) and the *pop-1* gene silenced via feeding-induced RNAi. Preliminary observations in *end-3(ok1448); pop-1(RNAi)* embryos showed that ectopic coelomocytes were made as well as ectopic pharynx muscle (Owraghi et al., 2009). In addition, laser ablation studies, in which the MS cell was ablated, confirmed that the E blastomere was responsible for producing the extra coelomocytes (Owraghi et al., 2009). These results proposed that the MS fate was being restored and that E was adopting an MS-like fate. Further experiments were followed-up with a strain that consisted of *end-1*and *end-3* deletions and *pop-1* being silenced via RNAi or the *pop-1(zu189)* mutation was introduced, particularly in the strain carrying the

pharynx muscle marker *myo-2*::GFP. Similar, but more penetrant, results were produced in which the E blastomere, in addition to the MS blastomere, was making coelomocytes as well as other MS-derived tissues (pharynx, pharynx muscle, and body wall muscle). These findings were not expected and the hypothesis was that these triple mutants would result in ectopic C-like tissues (epidermis), since the C-specific regulator, PAL-1, is present in both MS and E cells. Therefore, it is possible that, in addition to MS-like tissues being produced in the triple mutant embryos, C-like tissues would be made, as well. Therefore, the epidermal marker, *nhr-25*::YFP, was used to determine whether the MS and or E blastomeres produce C-derived epidermis, as well. Laser isolation studies done in the triple mutant embryos confirmed that the MS cell did not make epidermis, while the E blastomere produced epidermis in 9/11 embryos.

Since the E blastomere in *end-1,3; pop-1* triple mutant embryos produces both MS- and C-like tissues it was not obvious whether or not the E cell was making both types of tissues at the same time or if the cell made a random decision to make one type of tissue over the other. Therefore a new strain was constructed in which the *end-1,3; pop-1(RNAi)* mutant embryos were expressing both the *cup-4*::GFP marker as well as the *nhr-25*::YFP marker. The fluorescent markers were distinguished using different fluorescent filters. It was found, through laser isolation studies, that these embryos made both coelomocytes and epidermis at the same time, suggesting that the E blastomere was adopting a mixed MS- and C-like fate in the absence of *end-1,3* and *pop-1*.

Finally, the next question was whether or not the MS-like tissues that were produced by the E cell in the triple mutants was due to the same SKN-1/MED-1,2 gene

regulatory pathway involved in MS specification. Therefore, quadruple mutant animals were constructed in which *end-1, end-3, tbx-35,* and *ceh-51* were depleted. The *pop-1* gene was, again, silenced via feeding-induced RNAi, resulting in *tbx-35; ceh-51, end-1,3; pop-1(RNAi)* mutant embryos. These embryos failed to produce coelomocytes as well as other MS-specific tissues, suggesting that the ectopic MS-derived tissues produced by the E blastomere in the triple mutant embryos are in fact made through the normal MS-specification pathway.

These results reveal that Wnt-signaled POP-1 and END-1,3 actively repress MS specification in E, perhaps through another Wnt-dependent mechanism to enforce endodermal fate. As previously mentioned, POP-1 is not modified in the MS cell by the Wnt/β-catenin asymmetry pathway and the *end-1,3* genes are repressed by POP-1 allowing the MED factors to activate *tbx-35* and *ceh-51* (Broitman-Maduro et al., 2006; Broitman-Maduro et al., 2009). Furthermore, the findings suggest that the blastomere specification pathways display a phenotype prevalence revealed by various mutant backgrounds. In the absence of *pop-1* the two EMS daughters adopt an E-like fate, while elimination of *end-1*, *end-3* and *pop-1*results in two MS-like cells as a result of TBX-35 and CEH-51 activity. However, when *tbx-35*, *ceh-51*, *end-1*, *end-3*, and *pop-1* are absent both MS and E adopt a C-like fate, due to the presence of PAL-1. Taken together, this reveals a hierarchy of E>MS>C fates. This is actually similar to the posterior prevealance pattern of the Hox genes, in which the most posteriorly expressed Hox gene controls regional identity when multiple Hox genes are forcibly coexpressed, although

the mechanism in which this occurs is different than in *C. elegans* (Capovilla and Botas, 1998; Yekta et al., 2008).

Combinatorial control of cell fate specification

The collective work presented in the preceding chapters demonstrates the importance of combinatorial control in cell fate specification. Similar to the many signaling pathways that overlap and crosstalk to give rise to a desired output within a cell (i.e., production of secretory hormones, cell division, immune response, etc.), early cells of a developing embryo undergo cell-cell interactions or consist of maternal determinant factors that initiate the activation of zygotic transcription factors to regulate the fate of a particular cell. It is through a combination of multiple regulators that specify a desired cell fate within the early embryo. For instance, the specification of MS and E require a combinatorial control mechanism that involves two maternal pathways to give rise to the activation of lineage-specific blastomere identity factors (Maduro, 2010). The SKN-1 maternal factor specifies the fate of the EMS blastomere and activates the zygotic GATA factors, MED-1,2, while the WNT/β-catenin pathway makes the EMS daughter cells different from each other through the activity of TCF/POP-1.

As previously described the SKN-1 transcription factor binds to MED-1 and MED-2 (MED-1,2) binding sites and activates them in the EMS blastomere. The activity of MED-1,2 results in activation of the MS-specific factor, TBX-35, which proceeds to activate downstream target genes that specify mesoderm-derived tissues. However, the NK-2 homeodomain factor, CEH-51, has been identified to be a target of TBX-35 and

also involved in proper development of MS-derived tissues (Broitman-Maduro et al., 2009). If the EMS cell is isolated from the embryo at the four-cell stage both EMS daughters will become MS-like, presumably because the MS-specific factors TBX-35 and CEH-51 are present and active in both EMS daughter cells. However, in the intact embryo this does not occur as a result of the activity of the nuclear effector TCF/POP-1. At the posterior pole of the four-cell embryo, the P_2 blastomere sends a Wnt signal to the EMS cell. The Wnt signal results in activation of β-catenin factors that regulate levels of TCF/POP-1 in the E cell and bind to TCF/POP-1 in the nucleus of the E cell to activate transcription of endoderm-specifying genes, *end-1* and *end-3*. POP-1 normally acts as a repressor in the absence of Wnt signal, but its repressive characteristic is modified in the E cell as a result of the Wnt signal, so that it can act as an activator. It is through this WNT signal provided by the P_2 cell that results in MS and E to become different. Thus, it is both, the maternal SKN-1 and the Wnt/β-catenin pathways that converge to specify the MS and E blastomeres.

The recent findings on the function of POP-1 in the MS blastomere demonstrate that its major role is to block activation of *end-1* and *end-3*. This was an important finding since it was a question as to whether or not POP-1 had any other function in the MS cell. Others had suggested that POP-1 acts with SKN-1 to specify the fate of MS (Lin et al., 1995). In addition, POP-1 has recently been found to have a positive role in MS-specification in the related nematode, *C. briggsae* (Lin et al., 2009). Therefore, through elimination of the activity of POP-1 and END-1,3 we were able to confirm that POP-1 primarily functions to block *end-1,3* in MS. Furthermore, the results suggest that POP-1

may also function in the E cell to block MS-specific genes either through activity of END-1 and END-3 or through another unidentified factor that is upstream of END-1,3 and downstream of POP-1, since the absence of *pop-1*, *end-1*, and *end-3* results in the E cell to adopt an MS-like fate. This hypothesis is proposed to be true in *C. briggsae* in which POP-1 blocks MS genes in the E cell in wild-type embryos, although this has not been directly tested (Lin et al., 2009). This possibility may be likely since a similar type of regulation is found in *Drosophila* in which new binding sites for TCF have been found and thus allow it to act as a repressor upon binding of the β-catenin-like factor Armadillo (Arm) (Blauwkamp et al., 2008). Thus, the function of POP-1 in *C. briggsae* may have become different through changes in *cis*-regulatory sites within the promoter region of MS- and E-specific genes, resulting in a different regulatory pathway compared to that of *C. elegans*. It would be interesting to determine if the same MS-specific genes in *C. elegans*, *tbx-35* and *ceh-51*, are expressed at the same time and place in the *C. briggsae* early embryo and if they are activated by *Cb*-POP-1 (*C. briggsae* POP-1).

These findings also shed light on possible ways in which stem cells may be specified. Indeed, it is the presence of specific transcription factors that can result in a particular fate to be specified. As demonstrated in the studies describe above, the activation of specific transcription factors can result in activation of subsequent transcription factors to ultimately give rise to a desired cell fate. The presence of these factors can be a programmed mechanism within the pluripotent cell, or possibly through a mutation that may occur within the cell caused by an environmental factor that could induce activation of a specification factor. The specification properties of stem cells can

be studied *in vitro* by introducing chemical factors in cell culture that result in specification of a particular fate or ultimately differentiation. A recent study demonstrated that the germ cell fate in the adult *C. elegans* can be chemically reprogrammed, while in the absence of treatment the particular cell type is not specified (Morgan et al., 2010). Thus, this type of finding could also present new ideas for regenerative medicine. In *C. elegans*, cells of the early embryo are specified very early as a result of the presence of maternal determinants (i.e., SKN-1, PAL-1, etc.), but even if these factors are not present the cells would ultimately differentiate. Thus it is difficult to study the properties of stem cells in *C. elegans*, but the cell fate specification pathways that are elucidated and studied in this model system can provide insight into how stem cells, in mammalian organisms for example, become committed to a specific fate.

Future work

Direct repression of MS-specification genes in the E lineage of C. elegans early embryo

Based on the observations obtained from *end-1,3(-); pop-1(-)* embryos suggests that POP-1, not only functions to block *end-1* and *end-3* in the MS cell, but also blocks the MS fate in the E cell. In addition, it is shown that *tbx-35*::GFP is expressed in the E lineage of *pop-1(RNAi)* embryos, while *ceh-51* transcripts are abundantly present in the E lineage of *end-1,3(-); pop-1(RNAi)* embryos (Broitman-Maduro et al., 2009; Owraghi et al., 2009). These findings lead to question whether END-1,3 act directly to block the MS-specific genes, *tbx-35* and *ceh-51* or if an unknown factor functions to block activation of MS-specific genes in the E lineage. To determine what factor is responsible

for inhibiting *tbx-35* and *ceh-51* in the E cell, a first approach is to identify the presence of binding sites for END-1,3 in the promoter region of *tbx-35* or *ceh-51*. If binding sites are present, chromatin-immunoprecipitation analysis can verify whether END-1 and/or END-3 directly bind to these promoter regions. In addition, gel-shift assays can be done to confirm the binding of END-1,3 factors to the promoter region of *tbx-35* and *ceh-51*. This will provide an idea that END-1,3 may be playing a role in the regulation of MS-specific genes in the E cell. However, it is also possible that END-1,3 may not be involved in regulating the MS-specific genes in the E cell, since in the absence of *end-1,3* the E cell adopts a C-like fate and not MS-like. Therefore, there must be an additional factor that depends on the activity of POP-1, and END-1,3 to inhibit activation of *tbx-35* and *ceh-51* in the E cell. One approach to identify this factor is to use Fluorescence Activated Cell Sorting (FACS) to isolate the E blastomere, expressing *ceh-51*::GFP for example, from *end-1,3(-); pop-1(-)* embryos and also isolate the MS cells from these embryos expressing *ceh-51*::GFP. A cDNA library can be made from both isolated blastomeres and ultimately sequenced to compare and screen for potential genes that may be active in the E blastomere and not active in the MS blastomere. Once this gene is identified, a mutant strain can be constructed to determine whether MS-specific genes are still expressed in the E lineage.

Comparison of regulation of mesoderm specification in C. elegans and C. briggsae

In *C. elegans*, it has been established that TBX-35 and CEH-51 are required for proper development of MS-specific tissues. To determine if these MS-specific factors

are evolutionarily conserved, one initial step is to identify the *tbx-35* and *ceh-51* homologous genes in the related nematode, *C. briggsae*, and observe their expression pattern in the early embryo. The next question to be asked is, do the mutant embryos fail to make MS-derived body wall muscle, posterior pharynx, and coelomocytes? Thus, *tbx-35* and/or *ceh-51* mutants can be constructed to observe the phenotype using MS-specific reporters. In addition, *C. briggsae tbx-35* (*Cb-tbx-35)* and *Cb-ceh-51* transgenes can be introduced in *Ce-tbx-35* and *Ce-ceh-51* mutants, respectively, and observe whether the mutant phenotype is rescued in the presence of the transgene. This will test the ability of the *Cb-tbx-35* or *Cb-ceh-51* transgene to complement the functional loss of the *C. elegans* genes. Furthermore, this type of analysis will also help determine whether the function of TBX-35 and CEH-51 are conserved between the two species. Similar studies were conducted to elucidate the role of *Cb-pop-1* and it was found that the function of POP-1 in *C. briggsae* was to activate MS-specific genes rather than repressing them, as it is the case in *C. elegans*. It may be helpful to first attack the question of the roles of MS-specific genes in *C. briggsae* and how they are regulated in order to provide possibilities of how the MS-specific genes are regulated in the E lineage of *pop-1; end-1,3 C. elegans* mutant embryos or vise versa.

Bibliography

2009. Stem Cell Basics: Introduction. *In* Stem Cell Information. National Institutes of Health, U.S. Department of Health and Human Services, Bethesda, MD.

Altun, Z.F., Hall, D.H. 2008. Handbook of C. elegans Anatomy. WormAtlas.

Amin, N.M., S.E. Lim, H. Shi, T.L. Chan, and J. Liu. 2009. A conserved Six-Eya cassette acts downstream of Wnt signaling to direct non-myogenic versus myogenic fates in the C. elegans postembryonic mesoderm. *Dev Biol*. 331:350-60.

Arnone, M.I., and E.H. Davidson. 1997. The hardwiring of development: organization and function of genomic regulatory systems. *Development*. 124:1851-64.

Asahina, M., T. Valenta, M. Silhankova, V. Korinek, and M. Jindra. 2006. Crosstalk between a nuclear receptor and beta-catenin signaling decides cell fates in the C. elegans somatic gonad. *Dev Cell*. 11:203-11.

Austin, J., and J. Kimble. 1987. glp-1 is required in the germ line for regulation of the decision between mitosis and meiosis in C. elegans. *Cell*. 51:589-99.

Azpiazu, N., and M. Frasch. 1993. tinman and bagpipe: two homeo box genes that determine cell fates in the dorsal mesoderm of Drosophila. *Genes Dev*. 7:1325-40.

Barker, N., P.J. Morin, and H. Clevers. 2000. The Yin-Yang of TCF/beta-catenin signaling. *Adv Cancer Res*. 77:1-24.

Baugh, L.R., A.A. Hill, J.M. Claggett, K. Hill-Harfe, J.C. Wen, D.K. Slonim, E.L. Brown, and C.P. Hunter. 2005a. The homeodomain protein PAL-1 specifies a lineage-specific regulatory network in the C. elegans embryo. *Development*. 132:1843-54.

Baugh, L.R., A.A. Hill, D.K. Slonim, E.L. Brown, and C.P. Hunter. 2003. Composition and dynamics of the Caenorhabditis elegans early embryonic transcriptome. *Development*. 130:889-900.

Baugh, L.R., J.C. Wen, A.A. Hill, D.K. Slonim, E.L. Brown, and C.P. Hunter. 2005b. Synthetic lethal analysis of Caenorhabditis elegans posterior embryonic patterning genes identifies conserved genetic interactions. *Genome Biol*. 6:R45.

Baumgardt, M., I. Miguel-Aliaga, D. Karlsson, H. Ekman, and S. Thor. 2007. Specification of neuronal identities by feedforward combinatorial coding. *PLoS Biol*. 5:e37.

Behrens, J., J.P. von Kries, M. Kuhl, L. Bruhn, D. Wedlich, R. Grosschedl, and W. Birchmeier. 1996. Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature*. 382:638-42.

Bhattacharyya, A., Haeupler, B. 2009. Robust Regulatory Networks.

Bienz, M. 2005. beta-Catenin: a pivot between cell adhesion and Wnt signalling. *Curr Biol*. 15:R64-7.

Bissen, S.T., Ho, R.K., Weisblat, D.A. 2005. Cell interactions in the developing leech embryo. *BioEssays*. 4:152-157.

Blair, S.S. 1983. Blastomere ablation and the developmental origin of identified monoamine-containing neurons in the leech. *Dev Biol*. 95:65-72.

Blauwkamp, T.A., M.V. Chang, and K.M. Cadigan. 2008. Novel TCF-binding sites specify transcriptional repression by Wnt signalling. *EMBO J*. 27:1436-46.

Bodmer, R. 1993. The gene tinman is required for specification of the heart and visceral muscles in Drosophila. *Development*. 118:719-29.

Bowerman, B. 1995. Determinants of blastomere identity in the early C. elegans embryo. *Bioessays*. 17:405-14.

Bowerman, B. 1998. Maternal control of pattern formation in early Caenorhabditis elegans embryos. *Curr Top Dev Biol*. 39:73-117.

Bowerman, B., B.W. Draper, C.C. Mello, and J.R. Priess. 1993. The maternal gene skn-1 encodes a protein that is distributed unequally in early C. elegans embryos. *Cell*. 74:443-52.

Bowerman, B., B.A. Eaton, and J.R. Priess. 1992. skn-1, a maternally expressed gene required to specify the fate of ventral blastomeres in the early C. elegans embryo. *Cell*. 68:1061-75.

Brannon, M., M. Gomperts, L. Sumoy, R.T. Moon, and D. Kimelman. 1997. A beta-catenin/XTcf-3 complex binds to the siamois promoter to regulate dorsal axis specification in Xenopus. *Genes Dev*. 11:2359-70.

Brenner, S. 1974. The genetics of Caenorhabditis elegans. *Genetics*. 77:71-94.

Brody, T. 2005. The Interactive Fly: H15 and midline. Vol. 2010.

Broitman-Maduro, G., K.T. Lin, W.W. Hung, and M.F. Maduro. 2006. Specification of the C. elegans MS blastomere by the T-box factor TBX-35. *Development*. 133:3097-106.

Broitman-Maduro, G., M.F. Maduro, and J.H. Rothman. 2005. The noncanonical binding site of the MED-1 GATA factor defines differentially regulated target genes in the C. elegans mesendoderm. *Dev Cell*. 8:427-33.

Broitman-Maduro, G., M. Owraghi, W.W. Hung, S. Kuntz, P.W. Sternberg, and M.F. Maduro. 2009. The NK-2 class homeodomain factor CEH-51 and the T-box factor TBX-35 have overlapping function in C. elegans mesoderm development. *Development*. 136:2735-46.

Brunner, E., O. Peter, L. Schweizer, and K. Basler. 1997. pangolin encodes a Lef-1 homologue that acts downstream of Armadillo to transduce the Wingless signal in Drosophila. *Nature*. 385:829-33.

Byerly, L., R.C. Cassada, and R.L. Russell. 1976. The life cycle of the nematode Caenorhabditis elegans. I. Wild-type growth and reproduction. *Dev Biol*. 51:23-33.

Cadigan, K.M., and R. Nusse. 1997. Wnt signaling: a common theme in animal development. *Genes Dev*. 11:3286-305.

Calvo, D., M. Victor, F. Gay, G. Sui, M.P. Luke, P. Dufourcq, G. Wen, M. Maduro, J. Rothman, and Y. Shi. 2001. A POP-1 repressor complex restricts inappropriate cell type-specific gene transcription during Caenorhabditis elegans embryogenesis. *EMBO J*. 20:7197-208.

Capovilla, M., and J. Botas. 1998. Functional dominance among Hox genes: repression dominates activation in the regulation of Dpp. *Development*. 125:4949-57.

Carlson, J.M., and J. Doyle. 2000. Highly optimized tolerance: robustness and design in complex systems. *Phys Rev Lett*. 84:2529-32.

Cavallo, R.A., R.T. Cox, M.M. Moline, J. Roose, G.A. Polevoy, H. Clevers, M. Peifer, and A. Bejsovec. 1998. Drosophila Tcf and Groucho interact to repress Wingless signalling activity. *Nature*. 395:604-8.

Chapman, D.L., and V.E. Papaioannou. 1998. Three neural tubes in mouse embryos with mutations in the T-box gene Tbx6. *Nature*. 391:695-7.

Chen, G., and A.J. Courey. 2000. Groucho/TLE family proteins and transcriptional repression. *Gene*. 249:1-16.

Chen, G., J. Fernandez, S. Mische, and A.J. Courey. 1999. A functional interaction between the histone deacetylase Rpd3 and the corepressor groucho in Drosophila development. *Genes Dev*. 13:2218-30.

Chervitz, S.A., L. Aravind, G. Sherlock, C.A. Ball, E.V. Koonin, S.S. Dwight, M.A. Harris, K. Dolinski, S. Mohr, T. Smith, S. Weng, J.M. Cherry, and D. Botstein. 1998. Comparison of the complete protein sets of worm and yeast: orthology and divergence. *Science*. 282:2022-8.

Clevers, H., and M. van de Wetering. 1997. TCF/LEF factor earn their wings. *Trends Genet*. 13:485-9.

Clonkin, E.G. 1905. Mosaic development in ascidian eggs. *Journal of Experimental Zoology*. 2:145-223.

Consortium, C.e.S. 1998. Genome sequence of the nematode C. elegans: a platform for investigating biology. *Science*. 282:2012-8.

Coroian, C., G. Broitman-Maduro, and M.F. Maduro. 2006. Med-type GATA factors and the evolution of mesendoderm specification in nematodes. *Dev Biol*. 289:444-55.

Cremazy, F., P. Berta, and F. Girard. 2000. Sox neuro, a new Drosophila Sox gene expressed in the developing central nervous system. *Mech Dev*. 93:215-9.

Crittenden, S.L., E.R. Troemel, T.C. Evans, and J. Kimble. 1994. GLP-1 is localized to the mitotic region of the C. elegans germ line. *Development*. 120:2901-11.

Daniels, D.L., and W.I. Weis. 2005. Beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation. *Nat Struct Mol Biol*. 12:364-71.

Davidson, E.H. 1991. Spatial mechanisms of gene regulation in metazoan embryos. *Development*. 113:1-26.

Davidson, E.H. 2009. Endomesoderm and Ectoderm Models.

Davidson, E.H., and M.S. Levine. 2008. Properties of developmental gene regulatory networks. *Proc Natl Acad Sci U S A*. 105:20063-6.

Davidson, E.H., J.P. Rast, P. Oliveri, A. Ransick, C. Calestani, C.H. Yuh, T. Minokawa, G. Amore, V. Hinman, C. Arenas-Mena, O. Otim, C.T. Brown, C.B. Livi, P.Y. Lee, R. Revilla, M.J. Schilstra, P.J. Clarke, A.G. Rust, Z. Pan, M.I. Arnone, L. Rowen, R.A. Cameron, D.R. McClay, L. Hood, and H. Bolouri. 2002. A provisional regulatory gene network for specification of endomesoderm in the sea urchin embryo. *Dev Biol*. 246:162-90.

De Renzis, S., J. Yu, R. Zinzen, and E. Wieschaus. 2006. Dorsal-ventral pattern of Delta trafficking is established by a Snail-Tom-Neuralized pathway. *Dev Cell*. 10:257-64.

Dierick, H., and A. Bejsovec. 1999. Cellular mechanisms of wingless/Wnt signal transduction. *Curr Top Dev Biol*. 43:153-90.

Dobrovolskaia-Zavadaskaa, N. 1927. [On the spontaneous necrosis of the tail in the newborn mouse and on the existence of a hereditary phenotype 'non-viable']. *C R Soc Biol*. 97:114-116.

Donald, D.L. 1997. C. elegans II: Introduction to C. elegans. Cold Spring Harbor Laboratory Press, New York. 1-17 pp.

Dufourcq, P., M. Victor, F. Gay, D. Calvo, J. Hodgkin, and Y. Shi. 2002. Functional requirement for histone deacetylase 1 in Caenorhabditis elegans gonadogenesis. *Mol Cell Biol*. 22:3024-34.

Duval, A., J. Gayet, X.P. Zhou, B. Iacopetta, G. Thomas, and R. Hamelin. 1999. Frequent frameshift mutations of the TCF-4 gene in colorectal cancers with microsatellite instability. *Cancer Res*. 59:4213-5.

Edgar, L.G., S. Carr, H. Wang, and W.B. Wood. 2001. Zygotic expression of the caudal homolog pal-1 is required for posterior patterning in Caenorhabditis elegans embryogenesis. *Dev Biol*. 229:71-88.

Egan, S.E., B. St-Pierre, and C.C. Leow. 1998. Notch receptors, partners and regulators: from conserved domains to powerful functions. *Curr Top Microbiol Immunol*. 228:273-324.

Eisenmann, D.M. 2005. Wnt signaling. *In* Wormbook. T.C.e.R. Community, editor. Wormbook.

Fagotto, F., N. Funayama, U. Gluck, and B.M. Gumbiner. 1996. Binding to cadherins antagonizes the signaling activity of beta-catenin during axis formation in Xenopus. *J Cell Biol*. 132:1105-14.

Fire, A., S. Xu, M.K. Montgomery, S.A. Kostas, S.E. Driver, and C.C. Mello. 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature*. 391:806-11.

Fisher, A.L., and M. Caudy. 1998. Groucho proteins: transcriptional corepressors for specific subsets of DNA-binding transcription factors in vertebrates and invertebrates. *Genes Dev*. 12:1931-40.

Force, A., M. Lynch, F.B. Pickett, A. Amores, Y.L. Yan, and J. Postlethwait. 1999. Preservation of duplicate genes by complementary, degenerative mutations. *Genetics*. 151:1531-45.

Franks, D.M., T. Izumikawa, H. Kitagawa, K. Sugahara, and P.G. Okkema. 2006. C. elegans pharyngeal morphogenesis requires both de novo synthesis of pyrimidines and synthesis of heparan sulfate proteoglycans. *Dev Biol*. 296:409-20.

Friedman, A., and N. Perrimon. 2007. Genetic screening for signal transduction in the era of network biology. *Cell*. 128:225-31.

Fukushige, T., T.M. Brodigan, L.A. Schriefer, R.H. Waterston, and M. Krause. 2006. Defining the transcriptional redundancy of early bodywall muscle development in C. elegans: evidence for a unified theory of animal muscle development. *Genes Dev*. 20:3395-406.

Gao, F., and E.H. Davidson. 2008. Transfer of a large gene regulatory apparatus to a new developmental address in echinoid evolution. *Proc Natl Acad Sci U S A*. 105:6091-6.

Giese, K., J. Cox, and R. Grosschedl. 1992. The HMG domain of lymphoid enhancer factor 1 bends DNA and facilitates assembly of functional nucleoprotein structures. *Cell*. 69:185-95.

Gilbert, S.F. 2000. Developmental Biology. *In* The Developmental Mechanics of Cell Specification. Sinauer Assoicates, Inc.

Goldstein, B. 1993. Establishment of gut fate in the E lineage of C. elegans: the roles of lineage-dependent mechanisms and cell interactions. *Development*. 118:1267-77.

Good, K., R. Ciosk, J. Nance, A. Neves, R.J. Hill, and J.R. Priess. 2004. The T-box transcription factors TBX-37 and TBX-38 link GLP-1/Notch signaling to mesoderm induction in C. elegans embryos. *Development*. 131:1967-78.

Greenwald, I. 1998. LIN-12/Notch signaling: lessons from worms and flies. *Genes Dev*. 12:1751-62.

Greenwald, I.S., P.W. Sternberg, and H.R. Horvitz. 1983. The lin-12 locus specifies cell fates in Caenorhabditis elegans. *Cell*. 34:435-44.

Gridley, T. 1997. Notch signaling in vertebrate development and disease. *Mol Cell Neurosci*. 9:103-8.

Gu, Y., N.A. Hukriede, and R.J. Fleming. 1995. Serrate expression can functionally replace Delta activity during neuroblast segregation in the Drosophila embryo. *Development*. 121:855-65.

Harfe, B.D., C.S. Branda, M. Krause, M.J. Stern, and A. Fire. 1998. MyoD and the specification of muscle and non-muscle fates during postembryonic development of the C. elegans mesoderm. *Development*. 125:2479-88.

Heasman, J., A. Crawford, K. Goldstone, P. Garner-Hamrick, B. Gumbiner, P. McCrea, C. Kintner, C.Y. Noro, and C. Wylie. 1994. Overexpression of cadherins and underexpression of beta-catenin inhibit dorsal mesoderm induction in early Xenopus embryos. *Cell*. 79:791-803.

Hedgecock, E.M., J.G. Culotti, D.H. Hall, and B.D. Stern. 1987. Genetics of cell and axon migrations in Caenorhabditis elegans. *Development*. 100:365-82.

Henderson, B.R., M. Galea, S. Schuechner, and L. Leung. 2002. Lymphoid enhancer factor-1 blocks adenomatous polyposis coli-mediated nuclear export and degradation of beta-catenin. Regulation by histone deacetylase 1. *J Biol Chem*. 277:24258-64.

Henderson, S.T., D. Gao, E.J. Lambie, and J. Kimble. 1994. lag-2 may encode a signaling ligand for the GLP-1 and LIN-12 receptors of C. elegans. *Development*. 120:2913-24.

Herrmann, B.G., S. Labeit, A. Poustka, T.R. King, and H. Lehrach. 1990. Cloning of the T gene required in mesoderm formation in the mouse. *Nature*. 343:617-22.

Hinman, V.F., and E.H. Davidson. 2007. Evolutionary plasticity of developmental gene regulatory network architecture. *Proc Natl Acad Sci U S A*. 104:19404-9.

Hird, S.N., and J.G. White. 1993. Cortical and cytoplasmic flow polarity in early embryonic cells of Caenorhabditis elegans. *J Cell Biol*. 121:1343-55.

Hiroi, Y., S. Kudoh, K. Monzen, Y. Ikeda, Y. Yazaki, R. Nagai, and I. Komuro. 2001. Tbx5 associates with Nkx2-5 and synergistically promotes cardiomyocyte differentiation. *Nat Genet*. 28:276-80.

Hope, I.A.e. 1999. C. elegans: A Practical Approach. Oxford University Press, New York. 2-15, 245-270 pp.

Horb, M.E. 2000. Patterning the endoderm: the importance of neighbours. *Bioessays*. 22:599-602.

Horb, M.E., and G.H. Thomsen. 1997. A vegetally localized T-box transcription factor in Xenopus eggs specifies mesoderm and endoderm and is essential for embryonic mesoderm formation. *Development*. 124:1689-98.

Horner, M.A., S. Quintin, M.E. Domeier, J. Kimble, M. Labouesse, and S.E. Mango. 1998. pha-4, an HNF-3 homolog, specifies pharyngeal organ identity in Caenorhabditis elegans. *Genes Dev*. 12:1947-52.

Howard, M.L., and E.H. Davidson. 2004. cis-Regulatory control circuits in development. *Dev Biol*. 271:109-18.

Hu, P.J. 2007. Dauer. *WormBook*:1-19.

Huang, S., P. Shetty, S.M. Robertson, and R. Lin. 2007. Binary cell fate specification during C. elegans embryogenesis driven by reiterated reciprocal asymmetry of TCF POP-1 and its coactivator beta-catenin SYS-1. *Development*. 134:2685-95.

Hunter, C.P., and C. Kenyon. 1996. Spatial and temporal controls target pal-1 blastomere-specification activity to a single blastomere lineage in C. elegans embryos. *Cell*. 87:217-26.

Hurlstone, A., and H. Clevers. 2002. T-cell factors: turn-ons and turn-offs. *Embo J*. 21:2303-11.

Hutter, H., and R. Schnabel. 1994. glp-1 and inductions establishing embryonic axes in C. elegans. *Development*. 120:2051-64.

Joutel, A., C. Corpechot, A. Ducros, K. Vahedi, H. Chabriat, P. Mouton, S. Alamowitch, V. Domenga, M. Cecillion, E. Marechal, J. Maciazek, C. Vayssiere, C. Cruaud, E.A. Cabanis, M.M. Ruchoux, J. Weissenbach, J.F. Bach, M.G. Bousser, and E. Tournier-Lasserve. 1996. Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. *Nature*. 383:707-10.

Kidd, A.R., 3rd, J.A. Miskowski, K.R. Siegfried, H. Sawa, and J. Kimble. 2005. A beta-catenin identified by functional rather than sequence criteria and its role in Wnt/MAPK signaling. *Cell*. 121:761-72.

Kimble, J., and P. Simpson. 1997. The LIN-12/Notch signaling pathway and its regulation. *Annu Rev Cell Dev Biol*. 13:333-61.

Kitano, H. 2003. Cancer robustness: tumour tactics. *Nature*. 426:125.

Korinek, V., N. Barker, P. Moerer, E. van Donselaar, G. Huls, P.J. Peters, and H. Clevers. 1998. Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. *Nat Genet*. 19:379-83.

Korswagen, H.C. 2002. Canonical and non-canonical Wnt signaling pathways in Caenorhabditis elegans: variations on a common signaling theme. *Bioessays*. 24:801-10.

Korswagen, H.C. 2007. Wnt signaling in C. elegans: New insights into the regulation of POP-1/TCF-mediated activation and repression. *Advances in Developmental Biology*. 17:95-110.

Krause, M., S.W. Harrison, S.Q. Xu, L. Chen, and A. Fire. 1994. Elements regulating cell- and stage-specific expression of the C. elegans MyoD family homolog hlh-1. *Dev Biol*. 166:133-48.

Kuhn, C., C. Wierling, A. Kuhn, E. Klipp, G. Panopoulou, H. Lehrach, and A.J. Poustka. 2009. Monte Carlo analysis of an ODE Model of the Sea Urchin Endomesoderm Network. *BMC Syst Biol*. 3:83.

Lam, N., M.A. Chesney, and J. Kimble. 2006. Wnt signaling and CEH-22/tinman/Nkx2.5 specify a stem cell niche in C. elegans. *Curr Biol*. 16:287-95.

Lambie, E.J., and J. Kimble. 1991. Two homologous regulatory genes, lin-12 and glp-1, have overlapping functions. *Development*. 112:231-40.

Levine, M., and E.H. Davidson. 2005. Gene regulatory networks for development. *Proc Natl Acad Sci U S A*. 102:4936-42.

Li, L., I.D. Krantz, Y. Deng, A. Genin, A.B. Banta, C.C. Collins, M. Qi, B.J. Trask, W.L. Kuo, J. Cochran, T. Costa, M.E. Pierpont, E.B. Rand, D.A. Piccoli, L. Hood, and N.B. Spinner. 1997. Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. *Nat Genet*. 16:243-51.

Lin, K.T., G. Broitman-Maduro, W.W. Hung, S. Cervantes, and M.F. Maduro. 2009. Knockdown of SKN-1 and the Wnt effector TCF/POP-1 reveals differences in endomesoderm specification in C. briggsae as compared with C. elegans. *Dev Biol*. 325:296-306.

Lin, R., R.J. Hill, and J.R. Priess. 1998. POP-1 and anterior-posterior fate decisions in C. elegans embryos. *Cell*. 92:229-39.

Lin, R., S. Thompson, and J.R. Priess. 1995. pop-1 encodes an HMG box protein required for the specification of a mesoderm precursor in early C. elegans embryos. *Cell*. 83:599-609.

Little, J.W., D.P. Shepley, and D.W. Wert. 1999. Robustness of a gene regulatory circuit. *EMBO J*. 18:4299-307.

Logan, C.Y., McClay, D.R. 1999. Lineages that give rise to endoderm and mesoderm in the sea urchin embryo. Academic Press, New York. 41-58 pp.

Logan, C.Y., J.R. Miller, M.J. Ferkowicz, and D.R. McClay. 1999. Nuclear beta-catenin is required to specify vegetal cell fates in the sea urchin embryo. *Development*. 126:345-57.

Logan, C.Y., and R. Nusse. 2004. The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol*. 20:781-810.

Maduro, M.F. 2006. Endomesoderm specification in Caenorhabditis elegans and other nematodes. *Bioessays*. 28:1010-22.

Maduro, M.F. 2009. Structure and evolution of the C. elegans embryonic endomesoderm network. *Biochim Biophys Acta*. 1789:250-60.

Maduro, M.F. 2010. Cell fate specification in the C. elegans embryo. *Dev Dyn*.

Maduro, M.F., G. Broitman-Maduro, I. Mengarelli, and J.H. Rothman. 2007. Maternal deployment of the embryonic SKN-1-->MED-1,2 cell specification pathway in C. elegans. *Dev Biol*. 301:590-601.

Maduro, M.F., R.J. Hill, P.J. Heid, E.D. Newman-Smith, J. Zhu, J.R. Priess, and J.H. Rothman. 2005a. Genetic redundancy in endoderm specification within the genus Caenorhabditis. *Dev Biol*. 284:509-22.

Maduro, M.F., J.J. Kasmir, J. Zhu, and J.H. Rothman. 2005b. The Wnt effector POP-1 and the PAL-1/Caudal homeoprotein collaborate with SKN-1 to activate C. elegans endoderm development. *Dev Biol*. 285:510-23.

Maduro, M.F., M.D. Meneghini, B. Bowerman, G. Broitman-Maduro, and J.H. Rothman. 2001. Restriction of mesendoderm to a single blastomere by the combined action of SKN-1 and a GSK-3beta homolog is mediated by MED-1 and -2 in C. elegans. *Mol Cell*. 7:475-85.

Mango, S.E., C.J. Thorpe, P.R. Martin, S.H. Chamberlain, and B. Bowerman. 1994. Two maternal genes, apx-1 and pie-1, are required to distinguish the fates of equivalent

blastomeres in the early Caenorhabditis elegans embryo. *Development*. 120:2305-15.

McGrew, L.L., K. Takemaru, R. Bates, and R.T. Moon. 1999. Direct regulation of the Xenopus engrailed-2 promoter by the Wnt signaling pathway, and a molecular screen for Wnt-responsive genes, confirm a role for Wnt signaling during neural patterning in Xenopus. *Mech Dev*. 87:21-32.

Medina, K.L., J.M. Pongubala, K.L. Reddy, D.W. Lancki, R. Dekoter, M. Kieslinger, R. Grosschedl, and H. Singh. 2004. Assembling a gene regulatory network for specification of the B cell fate. *Dev Cell*. 7:607-17.

Mello, C.C., B.W. Draper, M. Krause, H. Weintraub, and J.R. Priess. 1992. The pie-1 and mex-1 genes and maternal control of blastomere identity in early C. elegans embryos. *Cell*. 70:163-76.

Mello, C.C., B.W. Draper, and J.R. Priess. 1994. The maternal genes apx-1 and glp-1 and establishment of dorsal-ventral polarity in the early C. elegans embryo. *Cell*. 77:95-106.

Mello, C.C., C. Schubert, B. Draper, W. Zhang, R. Lobel, and J.R. Priess. 1996. The PIE-1 protein and germline specification in C. elegans embryos. *Nature*. 382:710-2.

Mickey, K.M., C.C. Mello, M.K. Montgomery, A. Fire, and J.R. Priess. 1996. An inductive interaction in 4-cell stage C. elegans embryos involves APX-1 expression in the signalling cell. *Development*. 122:1791-8.

Miller, D.M., 3rd, N.S. Desai, D.C. Hardin, D.W. Piston, G.H. Patterson, J. Fleenor, S. Xu, and A. Fire. 1999. Two-color GFP expression system for C. elegans. *Biotechniques*. 26:914-8, 920-1.

Miller, D.M., F.E. Stockdale, and J. Karn. 1986. Immunological identification of the genes encoding the four myosin heavy chain isoforms of Caenorhabditis elegans. *Proc Natl Acad Sci U S A*. 83:2305-9.

Minguillon, C., and M. Logan. 2003. The comparative genomics of T-box genes. *Brief Funct Genomic Proteomic*. 2:224-33.

Miskowski, J., Y. Li, and J. Kimble. 2001. The sys-1 gene and sexual dimorphism during gonadogenesis in Caenorhabditis elegans. *Dev Biol*. 230:61-73.

Mizumoto, K., and H. Sawa. 2007. Two betas or not two betas: regulation of asymmetric division by beta-catenin. *Trends Cell Biol*. 17:465-73.

Mohr, O.L. 1919. Character Changes Caused by Mutation of an Entire Region of a Chromosome in Drosophila. *Genetics*. 4:275-82.

Molenaar, M., E. Brian, J. Roose, H. Clevers, and O. Destree. 2000. Differential expression of the Groucho-related genes 4 and 5 during early development of Xenopus laevis. *Mech Dev*. 91:311-5.

Molenaar, M., M. van de Wetering, M. Oosterwegel, J. Peterson-Maduro, S. Godsave, V. Korinek, J. Roose, O. Destree, and H. Clevers. 1996. XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos. *Cell*. 86:391-9.

Morgan, C.T., M.H. Lee, and J. Kimble. 2010. Chemical reprogramming of Caenorhabditis elegans germ cell fate. *Nat Chem Biol*. 6:102-4.

Nagel, S., L. Venturini, G.K. Przybylski, P. Grabarczyk, C. Meyer, M. Kaufmann, K. Battmer, C.A. Schmidt, H.G. Drexler, M. Scherr, and R.A. Macleod. 2009. NK-like homeodomain proteins activate NOTCH3-signaling in leukemic T-cells. *BMC Cancer*. 9:371.

Naiche, L.A., Z. Harrelson, R.G. Kelly, and V.E. Papaioannou. 2005. T-box genes in vertebrate development. *Annu Rev Genet*. 39:219-39.

Nishida, H. 1992. Developmental potential for tissue differentiation of fully dissociated cells of the ascidian embryo. *Roux's Archives of Developmental Biology*. 201:81-87.

Nishida, H. 1997. Cell fate specification by localized cytoplasmic determinants and cell interactions in ascidian embryos. *Int Rev Cytol*. 176:245-306.

Nowak, M.A., M.C. Boerlijst, J. Cooke, and J.M. Smith. 1997. Evolution of genetic redundancy. *Nature*. 388:167-71.

Nusse, R., A. van Ooyen, D. Cox, Y.K. Fung, and H. Varmus. 1984. Mode of proviral activation of a putative mammary oncogene (int-1) on mouse chromosome 15. *Nature*. 307:131-6.

Ohno, S. 1970. Evolution by Gene Duplication. *In* Springer-Verlag, Berlin-Heidelberg-New York.

Okkema, P.G., and A. Fire. 1994. The Caenorhabditis elegans NK-2 class homeoprotein CEH-22 is involved in combinatorial activation of gene expression in pharyngeal muscle. *Development*. 120:2175-86.

Okkema, P.G., S.W. Harrison, V. Plunger, A. Aryana, and A. Fire. 1993. Sequence requirements for myosin gene expression and regulation in Caenorhabditis elegans. *Genetics*. 135:385-404.

Oliveri, P., D.M. Carrick, and E.H. Davidson. 2002. A regulatory gene network that directs micromere specification in the sea urchin embryo. *Dev Biol*. 246:209-28.

Oliveri, P., Q. Tu, and E.H. Davidson. 2008. Global regulatory logic for specification of an embryonic cell lineage. *Proc Natl Acad Sci U S A*. 105:5955-62.

Orsulic, S., and M. Peifer. 1996. Cell-cell signalling: Wingless lands at last. *Curr Biol*. 6:1363-7.

Overton, P.M., L.A. Meadows, J. Urban, and S. Russell. 2002. Evidence for differential and redundant function of the Sox genes Dichaete and SoxN during CNS development in Drosophila. *Development*. 129:4219-28.

Owraghi, M., G. Broitman-Maduro, T. Luu, H. Roberson, and M.F. Maduro. 2009. Roles of the Wnt effector POP-1/TCF in the C. elegans endomesoderm specification gene network. *Dev Biol*.

Patton, A., S. Knuth, B. Schaheen, H. Dang, I. Greenwald, and H. Fares. 2005. Endocytosis function of a ligand-gated ion channel homolog in Caenorhabditis elegans. *Curr Biol*. 15:1045-50.

Peifer, M., C. Rauskolb, M. Williams, B. Riggleman, and E. Wieschaus. 1991. The segment polarity gene armadillo interacts with the wingless signaling pathway in both embryonic and adult pattern formation. *Development*. 111:1029-43.

Pflugrad, A., J.Y. Meir, T.M. Barnes, and D.M. Miller, 3rd. 1997. The Groucho-like transcription factor UNC-37 functions with the neural specificity gene unc-4 to govern motor neuron identity in C. elegans. *Development*. 124:1699-709.

Pickett, F.B., and D.R. Meeks-Wagner. 1995. Seeing double: appreciating genetic redundancy. *Plant Cell*. 7:1347-56.

Pocock, R., J. Ahringer, M. Mitsch, S. Maxwell, and A. Woollard. 2004. A regulatory network of T-box genes and the even-skipped homologue vab-7 controls patterning and morphogenesis in C. elegans. *Development*. 131:2373-85.

Polakis, P. 2000. Wnt signaling and cancer. *Genes Dev*. 14:1837-51.

Powell, W. 2004. Development: differentiation and determination, Gambier, OH.

Priess, J.R., H. Schnabel, and R. Schnabel. 1987. The glp-1 locus and cellular interactions in early C. elegans embryos. *Cell*. 51:601-11.

Priess, J.R., and J.N. Thomson. 1987. Cellular interactions in early C. elegans embryos. *Cell*. 48:241-50.

Qian, L., and R. Bodmer. 2009. Partial loss of GATA factor Pannier impairs adult heart function in Drosophila. *Hum Mol Genet*. 18:3153-63.

Revilla-i-Domingo, R., P. Oliveri, and E.H. Davidson. 2007. A missing link in the sea urchin embryo gene regulatory network: hesC and the double-negative specification of micromeres. *Proc Natl Acad Sci U S A*. 104:12383-8.

Riese, J., X. Yu, A. Munnerlyn, S. Eresh, S.C. Hsu, R. Grosschedl, and M. Bienz. 1997. LEF-1, a nuclear factor coordinating signaling inputs from wingless and decapentaplegic. *Cell*. 88:777-87.

Rijsewijk, F., M. Schuermann, E. Wagenaar, P. Parren, D. Weigel, and R. Nusse. 1987. The Drosophila homolog of the mouse mammary oncogene int-1 is identical to the segment polarity gene wingless. *Cell*. 50:649-57.

Rocheleau, C.E., J. Yasuda, T.H. Shin, R. Lin, H. Sawa, H. Okano, J.R. Priess, R.J. Davis, and C.C. Mello. 1999. WRM-1 activates the LIT-1 protein kinase to transduce anterior/posterior polarity signals in C. elegans. *Cell*. 97:717-26.

Roose, J., M. Molenaar, J. Peterson, J. Hurenkamp, H. Brantjes, P. Moerer, M. van de Wetering, O. Destree, and H. Clevers. 1998. The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. *Nature*. 395:608-12.

Ruvkun, G., and O. Hobert. 1998. The taxonomy of developmental control in Caenorhabditis elegans. *Science*. 282:2033-41.

Ryan, K., and A.J. Chin. 2003. T-box genes and cardiac development. *Birth Defects Res C Embryo Today*. 69:25-37.

Schnabel, R., Priess, J.R. 1997. C. elegans II: Specification of cell fates in the early embryo. Cold Spring Harbor Laboratory Press, New York. 361-382 pp.

Schnabel, R., C. Weigner, H. Hutter, R. Feichtinger, and H. Schnabel. 1996. mex-1 and the general partitioning of cell fate in the early C. elegans embryo. *Mech Dev*. 54:133-47.

Scholars, W.S. C. elegans as a model system. Vol. 2009. Waksman Student Scholars.

Sethi, A.J., R.C. Angerer, and L.M. Angerer. 2009. Gene regulatory network interactions in sea urchin endomesoderm induction. *PLoS Biol*. 7:e1000029.

Seydoux, G., C.C. Mello, J. Pettitt, W.B. Wood, J.R. Priess, and A. Fire. 1996. Repression of gene expression in the embryonic germ lineage of C. elegans. *Nature*. 382:713-6.

Shaham (ed.), S. Methods in cell biology. *In* WormBook. T.C.e.R. Community, editor. WormBook.

Shankland, M. 1984. Positional determination of supernumerary blast cell death in the leech embryo. *Nature*. 307:541-3.

Sharma, R.P., and V.L. Chopra. 1976. Effect of the Wingless (wg1) mutation on wing and haltere development in Drosophila melanogaster. *Dev Biol*. 48:461-5.

Sherwood, D.R., and D.R. McClay. 1997. Identification and localization of a sea urchin Notch homologue: insights into vegetal plate regionalization and Notch receptor regulation. *Development*. 124:3363-74.

Shetty, P., M.C. Lo, S.M. Robertson, and R. Lin. 2005. C. elegans TCF protein, POP-1, converts from repressor to activator as a result of Wnt-induced lowering of nuclear levels. *Dev Biol*. 285:584-92.

Siegfried, E., E.L. Wilder, and N. Perrimon. 1994. Components of wingless signalling in Drosophila. *Nature*. 367:76-80.

Smalley, M.J., and T.C. Dale. 1999. Wnt signalling in mammalian development and cancer. *Cancer Metastasis Rev*. 18:215-30.

Smith, J. 2008. A protocol describing the principles of cis-regulatory analysis in the sea urchin. *Nat Protoc*. 3:710-8.

Standley, H.J., O. Destree, M. Kofron, C. Wylie, and J. Heasman. 2006. Maternal XTcf1 and XTcf4 have distinct roles in regulating Wnt target genes. *Dev Biol*. 289:318-28.

Stelling, J., U. Sauer, Z. Szallasi, F.J. Doyle, 3rd, and J. Doyle. 2004. Robustness of cellular functions. *Cell*. 118:675-85.

Stennard, F.A., M.W. Costa, D.A. Elliott, S. Rankin, S.J. Haast, D. Lai, L.P. McDonald, K. Niederreither, P. Dolle, B.G. Bruneau, A.M. Zorn, and R.P. Harvey. 2003. Cardiac T-box factor Tbx20 directly interacts with Nkx2-5, GATA4, and GATA5 in regulation of gene expression in the developing heart. *Dev Biol*. 262:206-24.

Stiernagle, T. 2006. Maintenance of C. elegans. *In* WormBook. T.C.e.R. Community, editor. WormBook.

Stringham, E.G., and E.P. Candido. 1993. Targeted single-cell induction of gene products in Caenorhabditis elegans: a new tool for developmental studies. *J Exp Zool*. 266:227-33.

Strome, S. 1986. Fluorescence visualization of the distribution of microfilaments in gonads and early embryos of the nematode Caenorhabditis elegans. *J Cell Biol*. 103:2241-52.

Sulston, J.E., E. Schierenberg, J.G. White, and J.N. Thomson. 1983. The embryonic cell lineage of the nematode Caenorhabditis elegans. *Dev Biol*. 100:64-119.

Tabara, H., A. Grishok, and C.C. Mello. 1998. RNAi in C. elegans: soaking in the genome sequence. *Science*. 282:430-1.

Thomas, J.H. 1993. Thinking about genetic redundancy. *Trends Genet*. 9:395-9.

Thorpe, C.J., A. Schlesinger, J.C. Carter, and B. Bowerman. 1997. Wnt signaling polarizes an early C. elegans blastomere to distinguish endoderm from mesoderm. *Cell*. 90:695-705.

Timmons, L., and A. Fire. 1998. Specific interference by ingested dsRNA. *Nature*. 395:854.

Tuch, B.E. 2006. Stem cells--a clinical update. *Aust Fam Physician*. 35:719-21.

Twyman, R. 2002a. Model organisms: Fish. Wellcome Trust Human Genome.

Twyman, R. 2002b. Model organisms: Frogs and chickens. Wellcome Trust Human Genome, London.

Twyman, R. 2002c. Model organisms: The fruit fly. Wellcome Trust Human Genome, London.

Twyman, R. 2002d. Model organsims: Yeast. Wellcome Trust Human Genome, London.

Ulloa-Montoya, F., C.M. Verfaillie, and W.S. Hu. 2005. Culture systems for pluripotent stem cells. *J Biosci Bioeng*. 100:12-27.

van de Wetering, M., R. Cavallo, D. Dooijes, M. van Beest, J. van Es, J. Loureiro, A. Ypma, D. Hursh, T. Jones, A. Bejsovec, M. Peifer, M. Mortin, and H. Clevers. 1997. Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. *Cell*. 88:789-99.

Veeman, M.T., J.D. Axelrod, and R.T. Moon. 2003. A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. *Dev Cell*. 5:367-77.

Waltzer, L., and M. Bienz. 1999. The control of beta-catenin and TCF during embryonic development and cancer. *Cancer Metastasis Rev*. 18:231-46.

Wang, Y., P.N. Schnegelsberg, J. Dausman, and R. Jaenisch. 1996. Functional redundancy of the muscle-specific transcription factors Myf5 and myogenin. *Nature*. 379:823-5.

Wattler, S., A. Russ, M. Evans, and M. Nehls. 1998. A combined analysis of genomic and primary protein structure defines the phylogenetic relationship of new members if the T-box family. *Genomics*. 48:24-33.

Wiegner, O., and E. Schierenberg. 1999. Regulative development in a nematode embryo: a hierarchy of cell fate transformations. *Dev Biol*. 215:1-12.

Wilkinson, H.A., K. Fitzgerald, and I. Greenwald. 1994. Reciprocal changes in expression of the receptor lin-12 and its ligand lag-2 prior to commitment in a C. elegans cell fate decision. *Cell*. 79:1187-98.

Wood, W.B. 1988. The nematode Caenorhabditis elegans: Introduction to C. elegans Biology. Cold Spring Harbor Laboratory Press, New York. 1-16 pp.

Wylie, T., J.C. Martin, M. Dante, M.D. Mitreva, S.W. Clifton, A. Chinwalla, R.H. Waterston, R.K. Wilson, and J.P. McCarter. 2004. Nematode.net: a tool for navigating sequences from parasitic and free-living nematodes. *Nucleic Acids Res*. 32:D423-6.

Yekta, S., C.J. Tabin, and D.P. Bartel. 2008. MicroRNAs in the Hox network: an apparent link to posterior prevalence. *Nat Rev Genet*. 9:789-96.

Yochem, J., and I. Greenwald. 1989. glp-1 and lin-12, genes implicated in distinct cell-cell interactions in C. elegans, encode similar transmembrane proteins. *Cell*. 58:553-63.

Zhang, J., and M.L. King. 1996. Xenopus VegT RNA is localized to the vegetal cortex during oogenesis and encodes a novel T-box transcription factor involved in mesodermal patterning. *Development*. 122:4119-29.

Zhao, G., and J.B. Skeath. 2002. The Sox-domain containing gene Dichaete/fish-hook acts in concert with vnd and ind to regulate cell fate in the Drosophila neuroectoderm. *Development*. 129:1165-74.

Zhu, J., R.J. Hill, P.J. Heid, M. Fukuyama, A. Sugimoto, J.R. Priess, and J.H. Rothman. 1997. end-1 encodes an apparent GATA factor that specifies the endoderm precursor in Caenorhabditis elegans embryos. *Genes Dev*. 11:2883-96.