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Conditional Specification of Endomesoderm

By

David R. McClay^{a*}, Jenifer C. Croce^b and Jacob F. Warner^c

^a Department of Biology, Duke University, Durham, NC, USA

^b Sorbonne Université, CNRS, Laboratoire de Biologie du Développement de Villefranche-sur-Mer (LBDV), Institut de la Mer de Villefranche, Villefranche-sur-Mer, France

^c Department of Biology, University of North Carolina, Wilmington, NC, USA

* Corresponding author:

David R. McClay
Department of Biology
130 Science Dr. Box 90338
Duke University
Durham, NC 27708 USA
dmccclay@duke.edu

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Abstract

Early in animal development many cells are conditionally specified based on observations that those cells can be directed toward alternate fates. The endomesoderm is so named because early specification produces cells that often have been observed to simultaneously express both early endoderm and mesoderm transcription factors. Experiments with these cells demonstrate that their progeny can be directed entirely toward endoderm or mesoderm, whereas normally they establish both germ layers. This review examines the mechanisms that initiate the conditional endomesoderm state, its metastability, and the mechanisms that resolve that state into definitive endoderm and mesoderm.

Introduction

Conditional specification has fascinated developmental biologists for more than a century. Early embryologists discovered “regulative development” based on the demonstrated ability of isolated blastomeres to rescue an entire embryo in some cases. Cells of the sea urchin embryo, for example, isolated at the 2-cell or 4-cell stage, regulate and replace the missing parts to produce a normal half-sized or quarter-sized larva (Driesch, 1892). Over time, experiments with many different embryos and cell types eventually led to the proposed existence of a conditionally specified state (as opposed to a committed state). This conditionality can be tested: if a cell or a group of cells is isolated or transferred to an ectopic location in the embryo, the outcome is a measure of their conditionality. If those cells continue to develop toward their original fate only, the cells were committed at the time of transfer. However, if the cells divert to an alternative fate(s), then at the time they were isolated those cells were conditionally specified. Importantly, the experiment also indicates that the environment surrounding the conditionally specified cell provides the necessary information to direct its fate.

Endomesoderm, by definition, is conditionally specified, and this state is broadly distributed in embryos across the animal kingdom. The duration of the endomesoderm state varies greatly, its onset and its resolution into the definitive endoderm and mesoderm also varies mechanistically, but there are a number of properties that are conserved in this process. In most embryos the endoderm vs mesoderm fates are influenced non-autonomously through cell-cell interactions, signals, or other environmental inputs. To gain an impression of how conditional specification works in embryos, this review examines the means by which cells enter the endomesoderm state and the mechanisms leading to eventual fate commitment.

Conditional specification of endomesoderm is distributed broadly in the animal kingdom.

In the nematode *Caenorhabditis elegans*, the EMS cell at the 4-cell stage is fated to produce both endoderm and mesoderm descendant cells (Fig. 1). So, for a short time period the EMS cell embodies an endomesoderm status in the rapidly developing worm. Shortly after its emergence, at second cleavage, the EMS cell begins receiving a Wnt signal from its posterior neighbor, the P2 cell, which will contribute to the segregation of the endoderm and mesoderm fates. By third cleavage, this Wnt signal causes an asymmetric increase in SYS-1 (β -catenin) accumulation in the most posterior daughter cell, the E cell. This causes a reduction of repressive nuclear POP-1 (TCF) activity in the E daughter cell, while repressive POP-1 remains at a highly active in the MS daughter cell (Park and Priess, 2003). The high concentration of POP-1 represses expression of *end1* and *end3* in the MS cell, thereby inhibiting endoderm establishment in the mesoderm-fated MS cell. POP-1 in the E cell is bound by β -catenin thereby converting the POP-1 repressor into an activator and directing that cell toward endoderm specification through transcriptional activation of *end1* and *end3* (Maduro and Rothman, 2002; Owrighi et al., 2010). *End1* and *End3*, the early activated genes in the E cell, are Gata transcription factors and serve as pioneers that activate downstream transcription factors in the endoderm lineage. The high level of POP-1 repression in the MS cell represses the endoderm fate, allowing maternal *Skn-1* to activate *med1* and *med2*, both pioneering Gata factors, that activate downstream mesodermal genes (Lowry et al., 2009; Owrighi et al., 2010). These early regulatory steps thus produce a very short endomesoderm status. The brevity of these earliest regulatory

steps is facilitated by maternal components that activate zygotic expression of different Gata factors in the E and MS cells and these drive endoderm and mesoderm specification, respectively.

An earlier study with *C. elegans* on the timing of endoderm cell fate decision demonstrates the temporal sequence of this regulation. Goldstein (1992) separated the EMS cell from the signaling P2 cell at different timepoints after completion of second cleavage. Approximately 5–6 min of cell contact (signaling) between the P2 and EMS cell, after completion of second cleavage, was necessary before a then isolated EMS cell produced an E cell at the next division. Since reception of a canonical Wnt signal results in nuclear accumulation of β -catenin, it is presumed that 5–6 min into the 4-cell stage provides the necessary time for enough β -catenin accumulation to effect the response in the E cell. A later study determined when the EMS cell loses its ability to respond to the P2 signal. The EMS cell was isolated before the signal from P2 was received, and subsequently was recombined with the P2 cell at different timepoints. The competence of the EMS cell to respond to the P2 Wnt signal was lost about 3 min before the subsequent division (third cleavage) (Goldstein, 1995). Given that the cleavage cycle at that time is about 15 min, the EMS cell is thus competent to receive the inducing signal for approximately 12 min (Goldstein, 1995). However, Goldstein (1995) also showed that the P2 cell continues to signal after the EMS cell divides into an E and a MS cell, highlighting that signal reception can continue for several minutes into third cleavage assuring accumulation of enough nuclear β -catenin to specify the E blastomere. In this way the conditionality of the EMS cell declines as the different Gata factors become activated in the E and the MS cell.

In hemichordates, Wnt signaling is also involved in the conditional status of the endomesoderm. In these animals, Wnt signaling induces the establishment of this conditional state rather than its separation (Darras et al., 2011) (Fig. 1). FoxA (an endoderm marker) and zic (a mesoderm marker) are co-expressed by the endomesoderm cells for a period of time, demonstrating the existence of that conditional endomesoderm state. Later, during gastrulation, a FGF8/17/18 signal, issued from the ectoderm, induces some of the endomesoderm cells to become mesoderm (Green et al., 2013). Augmented FGF8/17/18 causes the entire endomesoderm to be specified as mesoderm, while knockdown of FGF8/17/18 results in the expression of endoderm markers only (Green et al., 2013). Thus, the endomesoderm of hemichordates is conditionally specified from early cleavage until sometime during gastrulation. Current data however do not reveal how that conditionality is maintained or what happens normally in endomesoderm cells that do not receive the FGF8/17/18 signal, and presumably become endoderm. Further, while early Wnt signaling plays a role in the initiation of endomesoderm, the details of this process are still incomplete.

In the urochordate *Ciona intestinalis*, a tunicate, mesendoderm (here, as with each of the model embryos covered, we use the naming convention for either mesendoderm or endomesoderm, used in that model system) establishment again is triggered by Wnt signaling. This takes place at the 16-cell stage in four vegetal cells, the NNE lineage (Fig. 1). Subsequently, one cell division later, the vegetal-most progeny of these cells exhibits continued nuclear accumulation of β -catenin and become endoderm, while the animal progeny of these cells lose nuclear accumulation of β -catenin and become neural/notochord (i.e. ectoderm/mesoderm) (Hudson et al., 2013; Imai et al., 2016; Oda-Ishii et al., 2016). At the 16-cell stage, the mesendoderm cells express foxA, foxD, and fgf9/16/20 and knockdown experiments demonstrate that all three genes are necessary for the transient mesendoderm state (Hudson et al., 2016). The short-lived mesendoderm

state in *C. intestinalis* is similar to the EMS cell in *C. elegans*, in that the mesendoderm and EMS cells display a very short period of conditional specification. In addition, in both animals, the short-lived mesendoderm state deploys the same mechanism to exit the conditional state, since in the tunicate β -catenin binds to TCF7 target sites as a co-activator to initiate Gata factor expression in the cells fated to be endoderm. Coincidentally, β -catenin blocks the binding of the Gata.A factor onto the enhancer of a mesoderm specifying gene *zic-r-b* (Hudson et al., 2013; Imai et al., 2016). This dual effect hence leads to the establishment of endoderm. In the animal progeny of the NNE cells the absence of β -catenin enables activation of the Gata.A factor and development of mesoderm. Thus, in this tunicate it appears that β -catenin is involved both in specifying mesendoderm and also in the later separation of mesendoderm progeny into either endoderm or mesoderm, with asymmetrically positive β -catenin signaling favoring the endodermal fate. There is also evidence that asymmetric Nodal signaling is involved in the separation of endoderm and mesoderm (Shi and Levine, 2008). Daughters of the mesendoderm that receive Nodal inputs from bordering cells become mesoderm, the Nodal signal resulting in inhibition of MAPK signal transduction thereby allowing activation of mesoderm genes in these cells (Shi and Levine, 2008).

Lineage studies in zebrafish also reveal a mesendoderm population of cells during epiboly (Warga and Nusslein-Volhard, 1999; Kikuchi et al., 2004; Kimmel and Warga, 1988) (Fig. 1). When single cells were labeled at the margin of the gastrulating zebrafish, the progeny of some of those cells gave rise to endoderm while other progeny of that labeled cell became mesoderm, and this was true if a single cell was initially labeled as late as 40% epiboly. If a single cell was first labeled later than 40% epiboly, all progeny of that labeled cell became either mesoderm or endoderm, thus indicating that after 40% epiboly a mesendoderm state was no longer present. The presence of a mesendoderm state was further supported recently through single cell RNA-seq studies, which show that the trajectory of the ectoderm first diverges from a mesendoderm population before the progeny of this population then diverges into mesoderm and endoderm after a relatively brief dual state (Farrell et al., 2018; Wagner et al., 2018). Induction of mesendoderm in zebrafish, as in other vertebrates examined, requires TGF β signaling (Rodaway et al., 1999), with two genes, *cyclops* and *squint*, both Nodal related genes (Feldman et al., 1998) providing the inputs. Several transcription factors necessary for specification of both mesoderm and endoderm are activated by this induction and co-expressed in the same cells (Poulain and Lepage, 2002). Thus, at least for a short while, a mesendoderm gene regulatory network operates in some cells at the margin of the blastoderm. Shortly thereafter, as epiboly continues, the mesendoderm state diverges, and this has been attributed in part to FGF and Delta-Notch signaling (Kikuchi et al., 2004; van Boxtel et al., 2018). A recent study indicates that this separation involves an incoherent feed forward regulatory loop such that Nodal activates expression of *fgf* and *dusp4*. The marginal mesendoderm cells that express a higher level of *dusp4* repress FGF signal transduction and become endoderm. Mesendoderm cells more distant from the margin retain FGF signaling due to a low level of *dusp4* expression and as a consequence these more distant cells become mesoderm (van Boxtel et al., 2018). This signaling and regulatory sequence begins at the dome stage and is completed by 50% epiboly. Thus, the conditional specification of mesendoderm lasts for only about 1.5 h, though for individual cells the timing may vary since the conditionality is not synchronous for all cells in the population.

The early stages of amphibian development include an overlap between the endodermal and mesodermal domains leading to an area often referred to as the mesendoderm (Charney et al., 2017). Early cut and paste experiments demonstrated that

amphibian embryos are remarkably regulative, and these were highlighted by the famous organizer induction experiments by Spemann and Mangold (1924). At the molecular level, a compilation of genes expressed during early mesendoderm specification suggests that this regulatory state lasts from midblastula transition (MBT) until about stage 10 (hence between 4 to about 11 h post-fertilization). Perturbations of a number of those genes, expressed during this time frame, shift either mesoderm to endoderm or in the opposite direction (Henry and Melton, 1998; Zorn et al., 1999; White et al., 2002; Chiu et al., 2014; Shivdasani, 2002). It isn't clear whether mesendoderm cells exist that express transcription factors of both fates, or whether the networks are separately mesoderm or endoderm from the beginning, but retain a level of conditionality enabling external inputs to easily shift the networks toward the alternative fate. Vegetal cells isolated prior to the start of gastrulation and inserted into the blastocoel of host embryos do not demonstrate commitment to endoderm until the early gastrula stage (about stage 10.5) (Wylie et al., 1987). Thus, the extended period of regulative development is a period prior to gastrulation during which the mesendoderm is conditional, regardless of whether the GRN leans toward an endoderm or mesoderm fate. Nodal and VegT signals, from the underlying normally-fated endoderm, induce mesoderm, though it has been noted that Nodal also plays a dual role to specify endoderm (Loose and Patient, 2004; Zhang and Klymkowsky, 2007). In addition, genes activated that lead toward commitment of either endoderm or mesoderm include Sox, Gata, and Fox family of transcription factors, all considered to be pioneer factors (Iwafuchi-Doi and Zaret, 2016; Iwafuchi et al., 2020; Zaret, 2020; Zaret and Carroll, 2011; Takahashi and Yamanaka, 2006). A valuable asset of research on endomesoderm in *Xenopus* is the large number of regulatory genes that are already known, including a rich knowledge of GRN contributions (Charney et al., 2017; Loose and Patient, 2004).

In birds and mammals, mesendoderm formation is initiated in the epiblast (Tsakiridis et al., 2014) and by the time the cells enter the primitive streak both endoderm and mesoderm appear to be largely committed toward their separate fates (Nowotschin et al., 2019; Nowotschin and Hadjantonakis, 2020) (Fig. 1). Studies on embryonic stem cell progression have been used to model the molecular path toward endoderm and mesoderm fates of epiblast cells. The pluripotent state is activated by Nodal family members and is maintained by expression of Nanog (Vallier et al., 2009). Once the primitive streak is established the expression of several transcription factors has been reported to be essential both for the entry of mesendoderm progeny through the primitive streak and for the fate restrictions of these cells (Acloque et al., 2011; Stryjewska et al., 2017). Curiously two such transcription factors, Snail and Zeb2, are also known regulators of epithelial mesenchymal transition (Cano et al., 2000; Taube et al., 2010).

From the brief sampling of the literature, a survey across the animal kingdom indicates that a conditional state of endomesoderm exists for many embryos, even if for a very brief period of time. Establishment of that state frequently involves Wnt signaling, and in vertebrates Nodal signaling is the most common signal used, although in vertebrates there is evidence that an earlier Wnt/ β -catenin signal may also initiate the specification sequence (Larabell et al., 1997; Miller and Moon, 1997). Experiments in several organisms have demonstrated that once a cell is conditionally specified as endomesoderm, perturbations can push this cell entirely toward endoderm or entirely toward mesoderm. Further, once the endoderm and mesoderm fates are achieved, conditionality tends to be quickly terminated. A common feature of that termination is the activation of Gata and Fox factors, both known to be pioneer transcription factors that

open closed chromatin regions (Zaret and Carroll, 2011). FoxA, a gene involved in early specification of endoderm in many embryos throughout the animal kingdom, is a good example of a pioneer transcription factor as it is known to play an important role in opening chromatin to allow access to enhancer sites that then drive the expression of endoderm specification genes (Iwafuchi-Doi and Zaret, 2016).

Developmental plasticity in the sea urchin embryo

The regulative capacities of sea urchin embryos are well known and have been studied for well over 100 years. Following the pioneering observations of Driesch (Driesch, 1892), the remarkable experiments of Horstadius (1939) provided seminal insights into the regulatory capacity of these embryos. Horstadius isolated and recombined many different embryo fragments. These showed the regulative capacity of cells, their inductive properties including temporal competence, and a differential loss of plasticity as differentiated cell types emerged. These early findings set the stage for later cellular and molecular studies that began late in the twentieth century. But even before the molecular explosion of information, those classic studies provided a number of clues about endomesoderm. Horstadius showed that mesomeres (the cells originating in the animal hemisphere during early cleavage), if isolated after third cleavage could only produce ectoderm, while endomesoderm originated in the vegetal half embryo (Horstadius, 1939). Experimentally, however, Henry et al. (1989) showed that the position of the third cleavage plane actually matters in this determination. Indeed, if that cleavage plane occurs below the equator of the embryo, isolated animal halves now produce endoderm (in addition to ectoderm) with some frequency. This indicates that the maternal information, localized in the vegetal half-embryo is a major contributor to endomesoderm.

A later study asked whether endoderm or mesoderm cells, once in the archenteron, were irreversibly committed. At late gastrulation, pieces of the archenteron were removed and in response the embryos replaced the missing tissues (McClay and Logan, 1996). The replacement came from adjacent cell populations; i.e. if just the midgut was removed, cells of the foregut and hindgut shifted to the identity of midgut cells and reestablished the correct proportionality of the three gut parts. Likewise, if the non-skeletal mesoderm (NSM) cells were removed, presumptive endoderm replaced the NSM cells. In still other studies, it was further shown that if the skeletogenic mesoderm cells were removed, they were replaced by NSM cells (Ettensohn and McClay, 1988).

These experiments raised a different question however. Was this demonstrated plasticity a reflection of conditional specification, or was the replacement observed actually a demonstration of regeneration? And what is the difference between the two processes? To address this issue, animal halves (cells above the equator of the spherical embryo) were isolated at different times after fertilization and their capacity to replace vegetal tissues (i.e. endoderm and mesoderm) was examined (Cheng et al., 2014). If isolated at any time from the 16-cell to the hatched blastula (HB) stage, the animal caps produce only ectoderm, suggesting that by fourth cleavage, those cells already lose their regulative properties. Transplantation of one or up to four micromeres (the vegetal-most cells of the embryo) onto animal caps induced the development of a second axis and of endomesoderm (Cheng et al., 2014; Croce et al., 2011; Horstadius, 1939; Ransick and Davidson, 1993), demonstrating that a transitory regulative ability of the animal halves does exist. However, that inductive competence ends after 4th cleavage (Ransick and Davidson, 1993; Cheng et al., 2014). Subsequently, after a long refractory period, if animal

caps are isolated following the beginning of gastrulation, they are capable of replacing endoderm, with no additional input (Cheng et al., 2014). Morpholinos to either Hox11/13b or FoxA, two early endoderm specification factors, prevented the gastrula-stage animal halves from producing endoderm, indicating that replacement of endoderm likely involves similar pathways as the original specification of endoderm. But is that a demonstration of conditionality? These data were interpreted to indicate that the early regulative ability of germ layers likely owes its plasticity, at least in part, to conditional specification, while the late replacement ability is likely due to the onset of a regenerative capacity through cell-reprogramming. Whether the two properties, conditional specification and regenerative capacity, share aspects of their molecular machinery, is unknown.

Interestingly, skeletogenic mesoderm and non-skeletal mesenchyme (NSM) cells demonstrate properties similar to those just described. The micromeres, precursors of the skeletogenic mesoderm cells (also known as Primary Mesenchyme Cells (PMCs)), are known to be specified autonomously almost from the time of their origin at the 16-cell stage. Indeed, if 16-cell stage micromeres are isolated and put into culture, or transplanted to ectopic positions in the embryo, these cells autonomously produce skeletogenic mesoderm cells and these cells only (Okazaki, 1975), hence corroborating an early committed state for these cells. In addition, these cells never replace any other cell type in embryos depleted of any other germ layer. If they are forced to overexpress an NSM specifier, such as Gcm, prior to the 16-cell stage they can differentiate as NSM, but that forced expression has to occur before the seminal specification event, the so-called double repression gate, that initiates skeletal cell specification (Damle and Davidson, 2012; Oliveri et al., 2003; Revilla-i-Domingo et al., 2007).

While the micromeres are specified autonomously, if they are removed from the embryo at the 16-cell stage, NSM cells reprogram to replace them, however not immediately. The replacement doesn't take place until gastrulation (Cheng et al., 2014), outlining again, the existence of a long refractory period. Thus, in early cleavage the skeletogenic mesoderm cells as well as the animal pole cells depart from conditionality quite quickly, and much later in development the animal pole cells and the NSM cells are capable of cell fate changes following an extended refractory period. These cell fate change capacities, launched at gastrulation, are likely due to the onset of a regenerative ability to reprogram the cells. While the causal mechanisms behind that reprogramming property are still not understood, they are likely distinct from the mechanisms that maintain conditionality. By contrast, the endomesoderm appears early in cleavage and experiments demonstrate that it retains conditionality for an extended period of time relative to the ectoderm and skeletogenic cells.

Conditionally specified endomesoderm in the sea urchin

Over the past 20+ years a detailed gene regulatory network (GRN) has been constructed for early sea urchin development. Cells arising from the vegetal half of the embryo are specified as endomesoderm for a period of time before separating into the distinct endoderm and mesoderm lineages. The GRN underlying these events is supported by an extensive series of experiments and in some cases, detailed cis-regulatory analyses, thereby providing a number of insights into the onset, maintenance, and termination of the conditional endomesodermal state. The GRN that is assembled during the conditional period includes expression of mRNAs that will ultimately become part of either the

mesoderm or endoderm GRNs. The GRN that persists to become endoderm or mesoderm depends on the input each cell receives from its local environment.

To understand the onset of the conditionality, it is necessary to review a few of the early cleavage events. An unequal fourth cleavage results in four micromeres at the vegetal pole of the embryo and four macromeres above them. The next cleavage of the micromeres also is unequal and produces small and large micromeres with the large micromeres fated to be the skeletogenic mesoderm cells and the small micromeres fated to contribute to, or be, the primordial germ cells (Fresques et al., 2016; Juliano et al., 2010; Yajima and Wessel, 2012). As soon as the micromeres appear they accumulate nuclear β -catenin and activate expression of *pmar1* (Logan et al., 1999; Oliveri et al., 2002; Oliveri et al., 2003, 2008). *Pmar1* is a repressor that rapidly represses activation of a second repressor, *hesC* (Revilla-i-Domingo et al., 2007). One consequence of the *hesC* repression is that by 5th cleavage the micromeres initiate expression of *delta* (Sweet et al., 2002). As this occurs, the eight macromeres at 5th cleavage, the cells just above the micromeres (Fig. 2), exhibit nuclear accumulation of β -catenin, a necessary step in the activation of endoderm (Logan et al., 1999; Wikramanayake et al., 1998; Emily-Fenouil et al., 1998). At the same time, *Delta*, produced by the micromeres, begins signaling to those eight adjacent macromeres, each of which expresses maternal Notch (Sherwood and McClay, 1999), and the consequence is activation of *gcm*, a mesoderm-specific transcription factor (Ransick et al., 2002). Meanwhile, Wnt signaling results in those same cells expressing *eve*, and perhaps other endoderm transcription factors (Peter, 2010). Thus, by the end of the 5th cleavage to the early 6th cleavage the eight *Veg2* macromeres have become endomesoderm and express marker genes of both germ layers. Perturbation experiments showed that augmentation of β -catenin throughout the embryo causes all cells to become endoderm, while inhibition of β -catenin accumulation results in a completely animalized embryo that expresses ectoderm markers only (Emily-Fenouil et al., 1998; Logan et al., 1999; Wikramanayake et al., 1998). Later studies with a GFP tagged form of β -catenin further showed that the β -catenin is actively destroyed in the animal hemisphere (Weitzel et al., 2004), while its initial accumulation, at least in the micromere nuclei, appears to be due, not to a Wnt signal, but to absence of Axin from these cells (Sun et al., 2021). Axin normally promotes destruction of β -catenin to prevent its accumulation (Zeng et al., 1997), so this early accumulation of β -catenin likely occurs in the absence of a Wnt signaling input. Whether that mechanism also is involved in the increased nuclear β -catenin in macromere nuclei at 5th cleavage, or whether that accumulation is due to expression of *Wnt8* by micromeres at 4th cleavage (Wikramanayake et al., 2004), isn't known. Overexpression of *Wnt8* leads to an excess of endomesoderm, while knockdown of *Wnt8* expression eliminates or greatly reduces endomesoderm specification (Wikramanayake et al., 2004). These observations led to the hypothesis that *Wnt8* signaling either activates or augments β -catenin, depending on the amount of Axin present.

Perturbation experiments with *Delta* demonstrate that any macromere contacting micromeres initiates expression of the mesodermal transcription factor *gcm* (Croce and McClay, 2010), and *gcm* is a direct target of Notch signal transduction through SuH (Ransick and Davidson, 2006; Ransick et al., 2002). Thus, the Wnt pathway and the Delta-Notch pathway combine to activate endomesoderm starting late in 5th cleavage. In the sea urchin *Paracentrotus lividus*, similar experiments showed that the transmembrane receptor *Frizzled1/2/7*, likely activated by the ligand *Wnt6*, triggers the nuclear accumulation of β -catenin specifically in the macromeres at fifth cleavage (Lhomond et al., 2012), supporting the hypothesis that β -catenin nuclearization in the macromeres is

driven by a Wnt signal. The Frizzled1/2/7 signaling was further established as necessary for the early expression of some endomesoderm genes, such as *wnt8* and *blimp1*, in the vegetal progeny of the macromeres as well as subsequently for the expression of early endoderm genes, such as *foxA*, in their descendants (Lhomond et al., 2012). Thus, as seen in embryos from many other phyla, canonical Wnt signaling appears to establish the top of the endomesoderm gene regulatory network in the sea urchin and to subsequently promote endoderm development.

At sixth cleavage, an equatorial division separates the macromeres into an upper tier of Veg1 cells and a lower tier of Veg2 cells (Fig. 2). Starting at this stage and onward, the Veg2 cells remain in contact with the Delta-positive micromeres, while the Veg1 cells lose contact with Delta. From that point forward, the Veg1 cells continue specification toward endoderm, and also, depending on relative inheritance of maternal information (due to the position of the third cleavage plane), toward ectoderm (Logan and McClay, 1997). The Veg2 cells, continue contact with the delta expressing micromeres and this maintains expression of mesodermal genes in these cells. The Veg2 cells also continue to respond to Wnt signaling through β -catenin and TCF/lef to maintain early endoderm specification by out-competing Groucho repression of TCF/lef (Range et al., 2005). As a consequence, between 6th and 8th cleavage the Veg2 endomesoderm cells express both early endoderm genes such as *foxA* and *hox11/13b*, and early mesoderm genes such as *gcm* and *gatae* (Croce and McClay, 2010; Peter and Davidson, 2010; Lhomond et al., 2012; Materna et al., 2013), thereby demonstrating the endomesodermal state of these cells through this time period.

A number of experiments reveal the conditionality of that endomesoderm state during this time. Increased Wnt signaling increases endoderm (Logan et al., 1999; Wikramanayake et al., 1998; Wikramanayake et al., 2004; Lhomond et al., 2012), while increased Notch signaling expands mesoderm at the expense of endoderm (Sherwood and McClay, 1999). Decreased Notch signaling results in endoderm expansion at the expense of mesoderm (Sherwood and McClay, 1999), and decreased Wnt signaling diminishes both mesoderm and endoderm specification (Logan et al., 1999; Wikramanayake et al., 1998; Lhomond et al., 2012). Thus, the dual signaling from Wnt and from Delta appears to maintain the conditional state of endomesoderm for at least from 6th cleavage (60-cell stage) to 8th cleavage. Experiments showed that Delta signaling must be continuous in order to maintain the mesoderm gene expression in the endomesoderm (Croce and McClay, 2010), and a continuing Wnt signal likely is necessary to maintain expression of the endoderm genes during this time.

At 8th cleavage, an equatorial cell division separates the Veg2 cells into two distinct cell tiers, the lower Veg2 tier and the upper Veg2 tier (Fig. 2). The upper Veg2 cells, farther away from the vegetal pole, lose contact with the Delta-producing micromeres and extinguish mesoderm marker gene expression, while they maintain expression of the endoderm markers (Croce and McClay, 2010; Peter and Davidson, 2010). If a Delta-expressing micromere is introduced ectopically to continue the contact with the upper Veg2 cells beyond the 8th cleavage, the upper Veg2 cells in contact with that ectopic cell continue to be endomesoderm and become mesoderm (Croce and McClay, 2010) (Fig. 3). That experiment thus begged the question as to how long was the Delta-Notch signal necessary before cells commit to mesoderm. To address this question, a series of transplantation experiments used a fluorescently tagged micromere that was ectopically placed between the Veg1 and Veg2 cells at 6th cleavage (60-cell stage) before removing it later. When left in that position, at least until to the HB stage (9 h post-fertilization), both upper and lower Veg2 cells, remaining in contact with a Delta-

producing micromere, continued to express *gcm* beyond 8th cleavage (Fig. 3). In the experiment the ectopically placed micromere was later removed to reveal when the Veg2 cell was able to continue toward mesoderm differentiation in the absence of Delta. If the micromere was removed between 7th and 8th cleavage (~6.5 h post-fertilization), the upper Veg2 cells that had been expressing *gcm* for one or two cell cleavages, extinguished *gcm* expression and became endoderm. On the other hand, if the fluorescently tagged ectopic micromere was removed about an hour after 8th cleavage (that is to say after about 3 h of continuous contact with Delta), the upper Veg2 cells differentiated as mesoderm (Croce and McClay, 2010). The explanation for these results is understood through experiments showing establishment of a feedback regulatory circuit between the Delta-Notch signal and Gcm (Materna et al., 2013; Ransick and Davidson, 2012). Gcm expression is first seen early in 6th cleavage. Under continuing Delta signaling, Gcm activates *gatae* expression. If Delta signaling continues even longer, Gatae activates expression of *six1/2*. If Delta signaling lasts even longer, Six1/2 accumulates and feeds back to maintain active *gcm* expression. That feedback activation of *gcm* expression removes the requirement for continuing Delta signaling, and now establishes a mesodermal differentiation trajectory. This at least partially explains how the conditionality is terminated for cells that will become mesoderm. Additionally, it has been hypothesized that Notch signaling represses *hox11/13b* expression in endomesoderm thereby reducing the likelihood that Veg2 cells become endoderm (Sethi et al., 2012). Since it is thought that the endodermal contribution to endomesoderm is maintained by Wnt signaling, Sethi et al. (2012) also showed that Notch signaling reduced the expression of Wnt1, a reduction that may divert cells increasingly toward the mesodermal fate.

It has been proposed that the accumulation of FoxA in presumptive endoderm cells is required to exclude mesoderm by repressing *gcm* (Oliveri et al., 2006), and that accumulation of Gcm causes repression of *foxA* expression (Peter and Davidson, 2009). However, cis regulatory analyses of the *foxA* enhancer does not show the presence of a Gcm binding site (de-Leon and Davidson, 2010), nor does analysis of the *gcm* enhancer show the presence of a FoxA binding site (de-Leon and Davidson, 2010), so this previously proposed reciprocal repression, if present, is indirect.

Furthermore, the mechanism through which endoderm cells lose their endomesoderm conditionality is not fully resolved. It could be by default (simply loss of Delta input while continuing to receive a Wnt input), or there could be an active mechanism directing endodermal commitment. What is known is that immediately following the loss of direct Delta signaling at 8th cleavage, the upper Veg2 cells begin expressing the brachyury (*bra*) transcription factor (Croce et al., 2001; Gross and McClay, 2001). Cis-regulatory studies indicate that Hox11/13b, Otx, and Blimp1, all factors expressed in the endomesoderm, have an input into the regulatory region of *bra* (Peter and Davidson, 2010). Bra, in turn, binds to the enhancer of *foxA* (de-Leon and Davidson, 2010). FoxA is expressed in endomesoderm earlier than endodermal *bra*, but at a low level. If *bra* expression is perturbed, *foxA* expression is retained at that low expression level. When *bra* expression is unperturbed, however, *foxA* expression increases after 8th cleavage to a high level and *gcm* expression is extinguished (Oliveri et al., 2006). The high level of *foxA* expression also requires continuing expression of Hox11/13b, otx and blimp1, as well as β -catenin-TCF activity and the expression of their common target *bra* (de-Leon and Davidson, 2010; Oliveri et al., 2006; Peter and Davidson, 2010). Thus, it is likely that the loss of conditionality of the endoderm-fated cells is due to the dual loss of Delta signaling and the increased expression of *foxA*, with FoxA acting as a pioneer

transcription factor to open chromatin sites for further endoderm specification, while perhaps simultaneously, directly or indirectly inhibiting *gcm*.

A recent single cell RNA-seq analysis provides further insights into the endomesoderm state (Massri et al., 2021). Cells were sequenced at 18 time points through the first 24 h of *Lytechinus variegatus* development. A bioinformatic approach (Waddington Optimal Transport) revealed the molecular trajectory of the lineages. That analysis provided a view of the endomesoderm trajectory, and the later separation of endoderm and mesoderm. As expected, cells expressing *bra* diverged toward endoderm, while cells expressing *gcm* diverged toward mesoderm after the HB stage. The divergence, however, was not synchronous. Some cells expressed only endoderm or mesoderm markers soon after the HB stage, but many cells continued to express both endoderm and mesoderm markers for at least an additional 4 h (Massri et al., 2021). These data indicate that conditionality ends asynchronously and perhaps inputs other than retention or loss of Delta-Notch signaling have an influence on resolution of the endomesodermal state.

Conclusions

Conditionality of the endomesoderm across the animal kingdom reveals a number of shared properties that lead to that state, properties of the conditionality itself, and properties that commit cells to subsequent fates. The Wnt signaling pathway is almost universally used to help initiate endomesoderm specification with Nodal signaling also used in many deuterostomes. The duration of conditionality is variable, from minutes to a number of hours. Where it has been studied in detail, that conditionality ends asynchronously with some cells retaining a conditionally specified state up to hours longer than other cells in the same organism. Resolution of the endomesoderm state is almost always due to asymmetric reception or loss of a signal. And in reaching definitive mesoderm and endoderm, pioneer transcription factors are thought to open chromatin, thereby initiating the gene regulatory sub networks of the two distinct germ layers. Often, when exiting the endomesoderm state, transcriptional repressors actively repress network components of the opposite germ layer.

The sea urchin endomesoderm GRN exhibits most of these shared properties. The endomesoderm state is regulated by a metastable network that can be pushed in either direction, endoderm or mesoderm, by perturbation of relatively few factors. The mesodermal state is increasingly stabilized by acquisition of a feedback loop established in sea urchins by *Gcm*, *Gatae*, and *Six1/2*. The endodermal state is increasingly stabilized by loss of Delta-Notch signaling, and an increase in Brachyury-directed *foxA* expression which represses the mesoderm circuitry and opens downstream endodermal GRN targets. Some features of the sea urchin endomesoderm network appear however to be unique to echinoderms. For example, Delta-Notch signaling is a prominent component of mesoderm specification in the sea urchin while it is used less frequently in other deuterostomes.

It is unclear if there is a selective pressure on the existence of conditionally specified states during embryonic development. There could be many answers to this question, though one prominent idea is that conditionality provides a way for the embryo to adjust proportionality. With conditional specification it isn't necessary to program each and every cell for an exact fate. Rather, with later signaling the embryo can dictate via signaling the correct proportion of mesoderm and endoderm to the embryo. As mentioned earlier in this review, the third cleavage of the sea urchin embryo is variable in providing a larger or smaller amount of cytoplasm to the future endomesoderm. The

ability to adjust the endomesoderm outcome through signaling, and the outcome of the Veg1 cells that contribute to endoderm plus a variable amount of ectoderm, gives the embryo the plasticity it needs to achieve a correctly proportioned larva.

The endomesoderm of the sea urchin embryo is only one example of a conditionally specified tissue. There are many instances during development in which cells within a germ layer later diverge into distinct fates. Perturbations prior to such a divergence often show that conditionality exists by pushing the cell fate in one direction or the other. Thus, there is a progression of conditional states of gene regulatory networks throughout development and the endomesoderm is simply an early example of many such metastable states in building a metazoan.

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Figure captions

Figure 1. Conditionally specified endomesoderm (or mesendoderm) shown in magenta across a selection of metazoan embryos as it resolves into endodermal lineages (yellow) and mesodermal lineages (red). In nematode embryos (A), the EMS cell gives rise to the MS and E cells (Ai), which specify mesoderm and endoderm respectively. In developing hemichordates (B), a conditionally specified endomesoderm later resolves into endoderm and mesoderm after gastrulation (Bi). Tunicate embryos at the 16-cell stage (C) exhibit high levels of nuclear β -catenin in the NNE lineage before it resolves into endoderm and neural-notochord (i.e. ectoderm/mesoderm) lineages (Ci). In fish embryos (D), during epiboly, ingressing hypoblast cells give rise to either endoderm or mesoderm (Di), suggesting a conditionally specified fate earlier. Mouse embryo epiblast (E) also exhibits cells with a potential to form endoderm or mesoderm (Ei), fates that become committed as cells pass through the primitive streak. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Figure 2. Conditional specification of endomesoderm in the sea urchin. At the 32-cell stage (A) the large micromeres (purple) first activate delta expression. The neighboring macromeres (magenta) display a high level of nuclear β -catenin that, along with the Delta input, initiates a conditionally specified endomesoderm. These signals persist through at least the 8th cleavage. (C) Eighth cleavage divides Veg2 cells into two tiers. The anterior progeny (the Veg2 upper tier) lose contact with the micromeres and therefore with the Delta signal thereby losing their mesodermal potential, while the Veg2 lower cells remain in contact with the Delta producing micromeres and adopt a mesodermal fate. (D) At the beginning of gastrulation, the micromeres undergo an epithelial-mesenchymal transition to become the PMCs, and the non-skeletal mesoderm will soon initiate invagination of the archenteron, followed by invagination of the Veg2 endoderm and finally the Veg1

endoderm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Figure 3. Delta-Notch signaling from the micromeres establishes mesoderm. Micromeres first appear at the 16-cell stage (A), activate delta expression at the 32-cell stage and begin expressing Delta at the 60-cell stage (Croce and McClay, 2010). (Ai) This signaling induces mesoderm gene expression in the adjacent endomesoderm (Veg2) cell layer (Ransick et al., 2002; Croce and McClay, 2010; Peter and Davidson, 2010). (Aii) Gcm, a mesoderm marker in the endomesoderm, is expressed at hatched blastula (HB) (reproduced from Croce and McClay, 2010). (B) If the micromeres are surgically removed at the 16-cell stage all endomesoderm is specified as endoderm only (Bi) because Delta is absent. (Bii) Consequently gcm is not expressed. (C) A single labeled micromere reintroduced at 16-cell stage in an otherwise micromere deficient embryo is sufficient to rescue mesoderm development and gcm expression (Ci, Cii). (D) If a micromere from a 16-cell stage embryo is transplanted to an ectopic location of a 60-cell stage wild-type embryo between the Veg1 and Veg2 layers that ectopic micromere induces ectopic mesoderm in the adjacent Veg2 lower and Veg2 upper tissues (Di, Dii). Embryos Aii, Bii, Cii and Dii are at the HB stage (8 to 9 h post-fertilization). They are all in lateral view with the animal pole to the top, except the embryo in Dii that is rotated to view the embryo from the vegetal pole to show the ring of mesoderm expressing gcm surrounding the endogenous and ectopic micromeres, which otherwise express T-brain, a micromere-descendant marker.

Figure 1

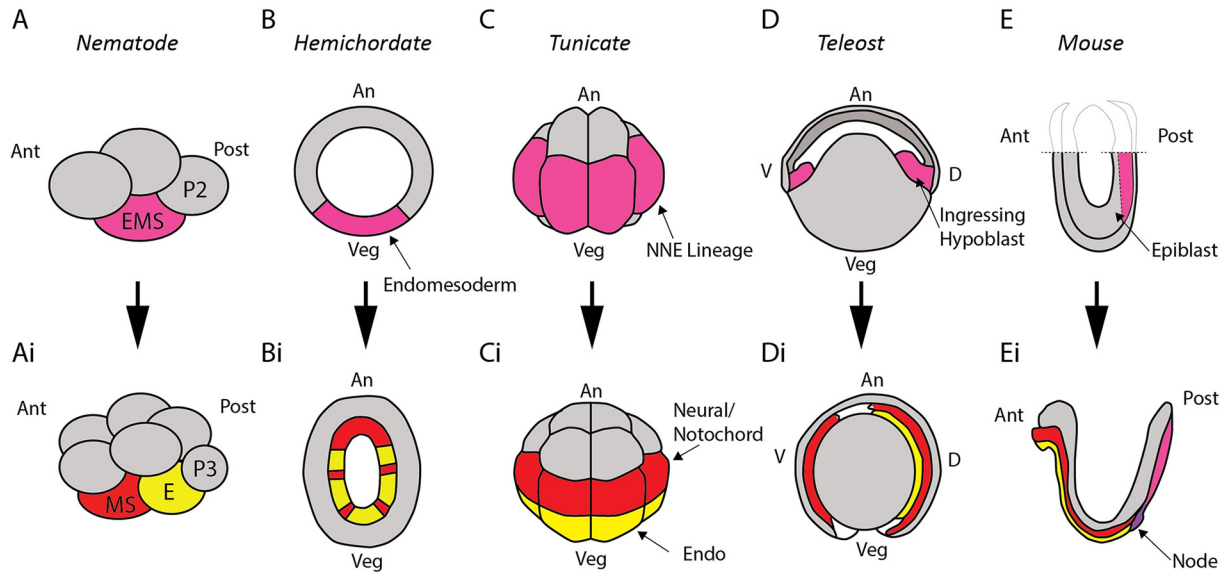


Figure 2

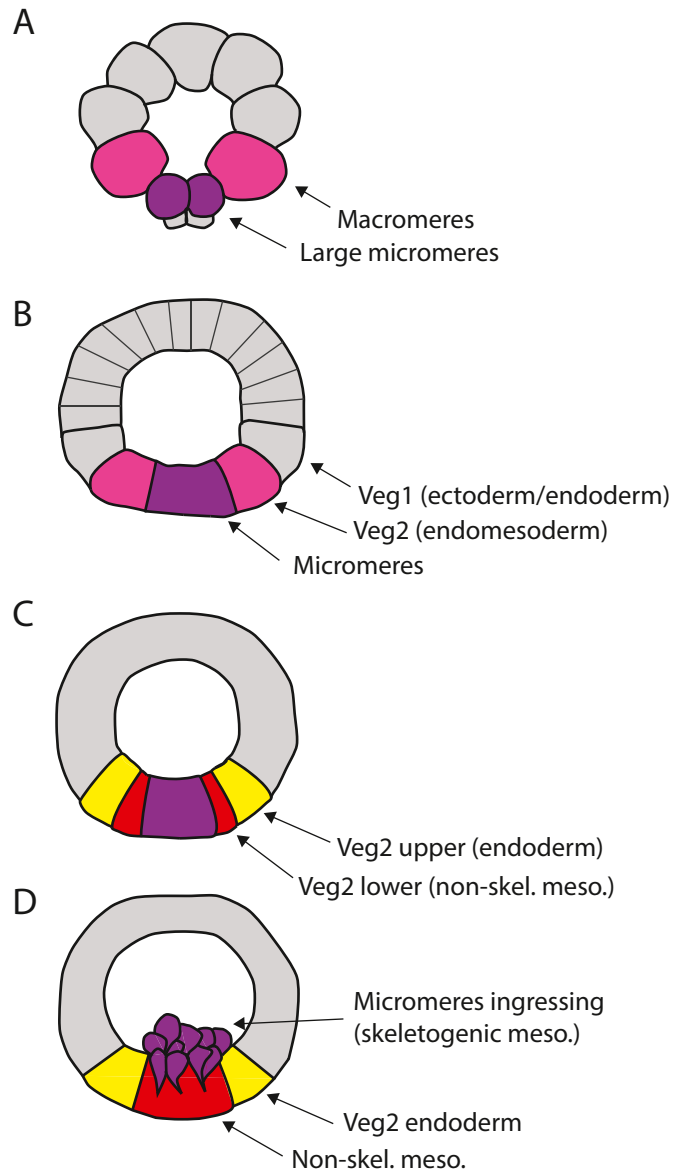


Figure 3

