A serpin is specifically expressed in and functionally required for ectomesoderm, a a hallmark of spiralian development

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Abstract

Among animals, diploblasts contain two germ layers, endoderm and ectoderm, while triploblasts have a distinct third germ layer called the mesoderm. Spiralians are a group of triploblast animals that have highly conserved development: they share the distinctive spiralian cleavage pattern as well as a unique source of mesoderm, the ectomesoderm. This population of mesoderm is distinct from endomesoderm and is considered a hallmark of spiralian development, but the regulatory network that drives its development is unknown. Here we identified ectomesoderm-specific genes in the mollusc *Tritia* (aka *Ilyanassa*) *obsoleta* through differential gene expression analyses comparing control and ectomesoderm-ablated embryos, followed by *in situ* hybridization of identified transcripts. We identified a *Tritia* serpin gene (*ToSerpin1*) that is specifically expressed in the ectomesoderm of the posterior and head. Ablation of the 3a and 3b cells, which make most of the ectomesoderm, abolishes *ToSerpin1* expression. Morpholino knockdown of *ToSerpin1* causes ectomesoderm defects, most prominently in the muscle system of the larval head. This is the first gene identified that is specifically implicated in spiralian ectomesoderm development.

Introduction

In gastrulation, diploblastic animals form two germ layers, endoderm and ectoderm, while triploblastic animals generate a third germ layer, the mesoderm. The mesoderm is a defining trait of bilaterians and makes tissues such as blood, bone and muscle. It has been proposed that the mesoderm played a decisive role for the diversification of body plans and structures in bilaterian evolution (Martindale et al. 2002; Technau and Scholz 2003).

In most bilaterians, at least some of the mesoderm is derived from a population of cells called the *endomesoderm,* because it also makes endodermal structures like gut (Rodaway and Patient 2001). In the large bilaterian clade called the Spiralia, mesoderm has a dual origin (Lillie 1895; Wilson 1898; Boyer et al. 1996). Like other bilaterians, spiralians have endomesoderm, which gives rise to most mesodermal structures, which in molluscs include the heart, kidney and main larval muscles. In spiralians, this cell lineage expresses some of the key factors known to be involved with endomesoderm specification in other bilaterians, such as *FoxA, Brachyury* and activated *β-catenin* (Perry et al. 2015), suggesting that spiralian endomesoderm is homologous with endomesoderm of other bilaterians.

The second source of mesoderm in spiralians, *ectomesoderm*, derives from cell lineages that otherwise give rise to ectoderm, and foregut in some systems (reviewed in Lambert 2008; Hejnol 2010). Ectomesoderm typically generates muscle, but the distinctive morphology of muscle cells means that they are easier to identify than other cell types, which has probably contributed to the impression that muscle is the dominant structure formed by ectomesoderm. It frequently includes non-muscle mesenchymal cells, and it has also been reported to make pigment cells in the head in the gastropod *Crepidula fornicata* (Osborne et al. 2018), and some non-muscle cells in the gut epithelium of *Capitella telata* (Meyer et al. 2010). Ectomesoderm has been found in all spiralian groups where it has been sought by lineage tracing, including molluscs, annelids, nemerteans,

flatworms and a phoronid (in molluscs: Dictus and Damen 1997; Render 1997; Hejnol et al. 2007; Chan and Lambert 2014; Lyons et al. 2015; in annelids: Huang 2002, Meyer et al. 2010; in a nemertean: Henry and Martindale 1996, 1998; in a flatworm: Boyer et al. 1996, 1998; in a phoronid: Freeman and Martindale 2002). Its presence has also been inferred by following cell populations using gene expression patterns in a brachiopod embryo (Passamaneck et al. 2015), and by examining the expression of conserved mesodermal genes the embryo of a bryozoan (Vellutini et al. 2017). This second source of mesoderm is considered a key aspect of the spiralian developmental program and an important contribution to spiralian bodyplan diversity (Boyer et al. 1998; Nielsen 2004; Lyons and Henry 2014; Osborne et al. 2018). Despite its likely importance, very little is known about the regulatory control of ectomesoderm development. The zinc finger protein *Tis11* is required for ectomesoderm development in *Tritia*, but it is also required for esophagus—the major non-ectomesoderm derivative of the lineages that generate ectomesoderm in this embryo, 3a and 3b (Chan and Lambert 2011). Several genes with mesodermal roles in other taxa have been reported in ectomesoderm, including orthologs of *Brachyury*, *Noggin*, *Goosecoid*, *FoxA*, *Twist*, and *Snail* (Lartillot et al, 2002a,b; Lespinet et al. 2002; Nederbragt et al. 2002; Perry et al. 2015; Passamaneck et al. 2015; Kozin et al. 2016). However, some of these genes are also expressed in other cell types, like endomesoderm (discussed below). For those that have been reported to be specific to ectomesoderm, this has not been conclusively determined. Many of the genes that are involved in epithelial to mesenchymal transition in dueterostomes are expressed in ectomesoderm cells in the mollusc *Crepidula fornicata*, suggesting that a similar gene regulatory network may drive this process in spiralians as well (Osborne et al. 2018).

Spiralians have a unique pattern of early development, called spiral cleavage, which is characterized by regularities in the proportion and direction of cell divisions. The regularity of spiral

cleavage allows the identification of all the cells in the blastula, including those that generate ectomesoderm. In spiral cleavage, the first two cell divisions generate four cells, called macromeres (named A, B, C and D). In the next three cleavage cycles, the macromeres then divide synchronously and asymmetrically towards the animal pole to generate tiers of four daughter cells with smaller size. Each set of these cells is called a quartet. In successive cleavages, the division angle lies on alternate sides of the animal-vegetal axis, generating a spiral pattern of daughter cells, which gives this mode of cleavage its name (Wilson 1898). Previous lineage-tracing and cell ablation studies have shown that spiralian endomesoderm arises from the fourth quartet daughter cell of the D quadrant, called 4d, while the ectomesoderm usually comes from 2nd and 3rd quartet daughters; in molluscs it often derives from the third quartet daughters of the A and B quadrants, 3a and 3b (Dictus and Damen 1997; Render 1997; Hejnol et al. 2007; Chan and Lambert 2014; Lyons et al. 2015).

The focus of this study is the gastropod *Tritia obsoleta* (a.k.a *Ilyanassa*). In this embryo, most of the ectomesoderm comes from 3a and 3b (Chan and Lambert, 2011, 2014). Lineage tracing and cell ablations (Clement 1986; Chan and Lambert 2011) indicate that anterior ectomesoderm cells from these clones make part of the muscle system in the velar lobes, which are ciliated structures on the side of the larval head used for swimming and feeding. The posterior ectomesoderm cells from 3a contribute to part of the larval retractor muscle, which is inside the shell but continuous with the velar musculature (see Figure 1, Chan and Lambert 2014; Lyons and Henry 2014). However, the exact roles of 3a and 3b ectomesoderm in the muscle system are still unclear. Two other cells contribute to ectomesoderm in this embryo. The 2b micromere contributes a muscle cell to the center of the head, and the 2c micromere contributes to muscle cells in the heart (Chan and Lambert 2014).

Here, we have determined the detailed structure of the velar musculature and examined the effect on this structure after ablation of 3a and 3b. We then compared the transcriptomes of embryos in which micromeres 3a and 3b had been ablated ("3a, 3b ablations") with control embryos to search for genes that are specifically expressed in ectomesoderm. 34 candidate ectomesoderm development genes recovered from that screen were examined by *in situ* hybridization to search for those with ectomesoderm-specific expression patterns. Further study of one of the candidate genes, a member of the serpin family we named *ToSerpin1*, showed that it is specifically expressed in ectomesoderm and is necessary for normal ectomesoderm development.

Results

The velar lobe musculature

Previous studies reported that both ectomesoderm and endomesoderm contribute to velar lobe muscles (Render 1997; Chan and Lambert 2014). Lineage tracing of 4d labels some muscles in the head, including some that appear to be radiating out to the periphery of the lobes, while tracing of 3a or 3b labels more of the radial muscles and also circumferential muscles around the periphery. The contribution of 3a, 3b and 4d to velar lobe muscle is also found in the gastropod *Crepidula fornicata* (Hejnol 2007; Lyons et al. 2012). For this study, we examined the velar musculature in detail with confocal microscopy (Figure 2). We observed two layers of muscle fibers radiating from the middle of the head to the outer part of the velar lobes; one layer of these fibers is on the anterior side of each velar lobe and one is on the posterior side. We call these radiating muscles. We also observed two muscles around the outer edge of each velar lobe; the anterior is thicker than the posterior one. We call these the anterior and posterior ring muscles (Figure 2A). Both sets of radiating muscles extend to the velar lobe's edge and connect to the corresponding ring muscle. This dual-layer structure was not reported in a previous description of the larval muscle system

(Evans et al. 2009). This could be due to incomplete sampling of development stages or different imaging approaches. From a functional morphology perspective, the two-layer structure makes sense, because it could generate the complex angular velar movements that are observed during swimming and feeding.

3a, 3b ablation and ectomesoderm-deficient embryos

To determine which parts of the velar lobe muscle require ectomesoderm, we ablated both of the cells that give rise to ectomesoderm: 3a and 3b. All velar lobes were smaller and had less complete muscle than wild-type and most were lacking both ring muscles (Table 1). In all but one, the anterior ring muscle was lacking; in the remaining lobe the anterior ring muscle was incomplete. In all but one lobe the posterior ring muscle was absent, and in the remaining lobe it was incomplete and thinner. In all lobes, the anterior layer of radiating muscle was largely gone, and the posterior layer was present but appeared disorganized. These results suggest that the 3a and 3b cell lineages are required for both of the velar lobe's ring muscles and the anterior layer of the radiating muscles, while the posterior radiating muscles can develop without the 3a or 3b derived ectomesoderm. Given the known contribution of 4d endomesoderm to head muscle including some radiating muscle fibers, this suggests that the posterior radiating muscles observed after 3a, 3b deletion are derived from 4d (Chan and Lambert 2014).

Screening for ectomesoderm-specific genes

To find genes that might be important for ectomesoderm development, we targeted a stage when the ectomesoderm lineages of 3a and 3b seem to be separating from the esophageal precursors and actively migrating internally away from ectoderm (between 48 and 56 hours A.E.L.; Chan and

Lambert 2014); we surmised that ectomesoderm-specific gene expression might begin at this stage. We reared 3a, 3b ablation and control embryos to this stage, and extracted RNA from three replicates with ten experimental and ten control embryos each. After sequencing, we performed differential gene expression analysis between experimental and control groups to search for differentially expressed genes. From this analysis we chose 34 genes to examine further (see Methods for details on how these were chosen).

To directly assay whether a putatively differentially regulated gene was specifically expressed in ectomesoderm, we performed *in situ* hybridization. In *Tritia*, most ectomesoderm comes from the 3a and 3b cell lineages, but these lineages also give rise to non-ectomesoderm structures such as esophagus and ectodermal cells in the velar lobe. Additionally, ablating 3a and 3b results in deficient digestive glands, even though these structures are derived from different lineages (Chan and Lambert 2011). Therefore, in this experiment, we not only expected to see ectomesoderm-specific genes, but also genes specific to the esophagus and digestive gland. Of the 34 genes we examined, two were expressed in the esophagus, and one was in the digestive gland and part of the esophagus (Figure 3A-C). As we had hoped, we also found three which appeared to have expression that was at least partly specific to the ectomesoderm. An Apolipoprotein-like gene was expressed in a pattern resembling the ectomesodermal components of the 3a and 3b lineages, but it was also strongly expressed in an internal structure that is not ectomesodermal (Figure 3D). A gene of unknown function was expressed in cells that appeared to be ectomesoderm, but it was also expressed in cells that seemed to be derived from the endomesodermal lineage (*ToMesoderm-expressed-1*; Figure 3E). Finally, we found a Serpin ortholog which appeared to be indistinguishable from the 3a and 3b ectomesoderm population (Figure 3F-J); we characterized this gene further below. We also recovered an ortholog of the transcription factor *Prospero*, which

appeared to be expressed in the developing nervous system (data not shown); all other transcripts we examined were broadly expressed or ubiquitous (sequences of these transcripts are in Supplemental Table 1).

ToSerpin1 as a potential ectomesoderm gene

We named the gene that appeared to be specific to the ectomesoderm *ToSerpin1*. Serpins are a superfamily of proteins, originally named after their main function as **ser**ine **p**rotease **in**hibitors (Huntington 2011). They share a conserved core domain consisting of three beta-sheets, 8-9 alpha-helices and an exposed region called the reactive center loop which interacts with their target protease (Gettins 2002; Huntington 2011). Their functions are not limited to the protease inhibition; serpins also play roles as hormone transporters and molecular chaperones (reviewed in Law et al. 2006). Importantly, some serpins have regulatory functions in development. For example, a serpin is required for normal dorsal-ventral axis patterning in *Drosophila* (Ligoxygakis et al. 2003). In *Xenopus*, a serpin suppresses mesoderm and promotes anterior development under the control of FGF signaling (Acosta et al. 2015).

The *ToSerpin1*-positive cells look like ectomesoderm cells that are labeled by lineage tracing of 3a or 3b, although their precise positions vary between embryos because they are migrating (Chan and Lambert 2014; see also Figure 4). In the embryo's posterior epithelium underlying the shell, the 3a and 3b-derived cells and *ToSerpin1*-positive cells are both flat with a mesenchyme morphology and similar distribution. With both labels, the anterior cells in the head are more rounded than the posterior cells. In both regions they are subectodermal, consistent with mesodermal identity.

To test if the *ToSerpin1* positive ectomesoderm-like cells do indeed come from 3a and 3b lineages, we ablated 3a and 3b and performed *ToSerpin1 in situ* hybridization. After 3a, 3b ablation, both anterior and posterior *ToSerpin1* positive ectomesoderm-like cells are not detected by *in situ* hybridization (Figure 4A-C). This is consistent with the idea that *ToSerpin* is expressed in the 3a and 3b derived ectomesoderm, but not the 2b or 2c ectomesodermal populations, or the 4d endomesoderm population. The simplest interpretation of the preceding ablation experiment is that *ToSerpin1* is only expressed in the 3a and 3b ectomesoderm, but there is an alternative model: since the lineage tracing data indicate that the 4d-derived cells mix somewhat with the ectomesodermal muscle progenitors in the head, it is possible that *ToSerpin1* is marking a population of cells that are derived from both of these lineages, but 3a and 3b-derived cells are necessary to induce *ToSerpin1* expression in the whole population. To examine this, we compared the number of cells between 3a-derived ectomesoderm cells and *ToSerpin1* positive cells, doubling the cell numbers from 3a lineage tracing embryos to account for the symmetrical but unlabeled 3b contribution (Chan and Lambert 2014). This takes advantage of the fact that the other populations of cells in the 3a and 3b lineages are easy to distinguish from the ectomesoderm: the small number of ectoderm cells that contribute to the back of the velum stay in the ectoderm, and esophageal precursors stay in a compact column deeper in the midline of the embryo (see Figure 4D-F). In both anterior and posterior subsets, the number of 3a lineage traced cells and *ToSerpin1* positive cell are indistinguishable statistically (Figure 4G). Together with the ablation results, these data are consistent with the idea that *ToSerpin1* is specifically labeling ectomesoderm cells. The single muscle cell contributed by 2b is also mixing with the other head ectomesoderm, but these cell counts do not rule out the contribution of single cell to the *ToSerpin1*-positive population.

ToSerpin1 morpholino knockdown

To test if *ToSerpin1* is required for ectomesoderm development, we injected a translation-blocking morpholino oligo (*ToSerpin1* MO1) into *Tritia* zygotes in a range of concentrations from 0.25mM to 1mM. At concentrations higher than 0.75mM, the embryos developed normally until the mid-organogenesis stage (3-4 day old) when they started to lose cells from the head and died within a few days. This is an unusual phenotype, we have not observed this with any of the ~40 morpholinos we have tested in this embryo previously (Lambert lab published and unpublished observations). The organogenesis lethality phenotype is consistent, reproducible, and is also caused by a second non-overlapping MO targeting *ToSerpin1* (discussed below). Concentrations below 0.5mM generated no detectable phenotype. We thus focused on injections at the 0.75mM concentration. The muscle system in the head was impaired after *ToSerpin1* MO1 injections compared to the control MO group (Figure 5 and Table 1). We observed a series of phenotypes in the velar lobe muscle system (Figure 5B, C), including smaller velar lobes, fewer radiating muscles, lack of anterior or posterior ring muscles, incomplete extension to ring muscle, thinner ring muscle and detachment of ring and radiating muscle. In addition, the distinct two-layer structure of radiating muscle disappeared in some larvae, making it hard to distinguish the two layers (Figure 5D, E). In sum, *ToSerpin1*MO1 knockdown phenotypes are generally similar to the velar muscle phenotypes generated by the 3a and 3b cell lineage ablation, though not as severe. Aside from the head defects, the larvae were generally wild-type. We often observed mild defects in the retractor muscle, though these were difficult to score conclusively. This is consistent with the contribution of 3a to a few strands of the retractor, and the previous report of 3a, 3b ablations, where 18/31 (58%) of cases had thinner or no retractor muscles (Chan and Lambert 2011, 2014).

257 To verify the specificity of the *ToSerpin1* MO1, we did two more sets of experiments. First,
258 we co-injected *ToSerpin1* MO1 along with a *ToSerpin* mRNA where the endogenous 5'UTR was
259 deleted so that the mRNA lacked the MO target site (Figure 5J and Table1). We found that the
260 overall phenotype was markedly less severe, indicating that the mRNA rescued the MO
261 knockdown. Second, we injected another non-overlapping *ToSerpin1* morpholino oligo, *ToSerpin1*
262 MO2. The phenotype of larvae injected with *ToSerpin1* MO2 larvae was similar in the kind and
263 severity of the defects observed, compared to that of MO1 (Figure 5F-I and Table 1). Taken
264 together, these results indicate that *ToSerpin1* is necessary for normal ectomesoderm development
265 in *Tritia*.
266

Discussion

Our approach, 3a, 3b ablation followed by RNA-seq and validation by *in situ* hybridization, uncovered six genes specifically expressed in 3a and 3b-dependent structures. Three of these had gene expression patterns consistent with ectomesoderm expression, including a gene of unknown function, an apolipophorin-like gene, and *ToSerpin1*. Using 3a, 3b ablation, followed by *ToSerpin1 in situ* hybridization, we showed that this gene is specific to the ectomesoderm. We characterized the main derivative of the ectomesoderm—the velar musculature of the larval head, and showed this is strongly disrupted after 3a, 3b ablation. Knockdown of *ToSerpin1* has a similar, but less severe effect than the ablations, indicating that this gene is required for normal development of the ectomesoderm. This is the first gene that has been functionally implicated specifically in spiralian ectomesoderm development.

Ectomesoderm has been considered one of the key aspects of the spiralian developmental program, and a potentially important innovation for body plan diversification of this clade (Boyer et al. 1996; Henry and Martindale 1999; Lambert 2008; Hejnol 2010; Osbourne et al. 2018). How and when did ectomesoderm evolve? It may have arisen in the spiralian common ancestor, perhaps from an ancestral condition with only endomesoderm. Alternatively, it may have evolved from a population of mesoderm in a protostome or bilaterian ancestor. Across bilaterians, many animal groups which have endomesoderm also have additional sources of mesoderm. In vertebrates, there is a population of anterior mesoderm called the prechordal plate, which serves as an organizer and contributes to the head (Seifert et al. 1993; Harland and Gerhart 1997). In ascidians, there is a definitive endomesodermal population, but mesoderm arises from other lineages as well (Nishida 1987; Hudson et al. 2016). In *C. elegans*, most of the mesoderm derives from the MS blastomere, which is a classic example of endomesoderm (Maduro 2010).

290 However, a number of muscle cells are derived from the anterior ABa lineage, and form part of
291 the pharanx (Sulston et al. 1983; Good et al. 2004; Priess 2005). In the shrimp *Sicyonia ingentis*,
292 there are also two origins of mesoderm, one that is associated with endoderm and arises from
293 teloblasts, and another, that has been called naupliar (i.e. larval), and only generates muscle
294 (Hertzler 2005; other similar patterns in crustaceans are reviewed in Gerberding et al. 2002). As
295 more is learned about the molecular basis of these non-endomesodermal mesodermal populations,
296 it will be interesting to learn if there are commonalities that could support the existence of such a
297 population in an older common ancestor. The homology of the spiralian ectomesoderm with
298 vertebrate prechordal plate has been previously proposed (Lartillot et al. 2002a). Their argument
299 was based on anatomical position and gene expression patterns, but there is an additional level of
300 similarity that has become apparent with subsequent studies. In *Patella vulgata*, the mollusc
301 studied by Lartillot et al., the 3a and 3b cells do not form esophagus as they do in other
302 gastropods, because its non-feeding larva does not have an esophagus. As a consequence, they did
303 not appreciate another parallel: molluscan 3a and 3b-derived ectomesoderm, like prechordal plate
304 mesoderm, derives from a lineage that also makes anterior endoderm. Regardless of whether
305 spiralian ectomesoderm evolved from a non-endomesodermal population in a protostome
306 common ancestor or evolved *de novo* in the spiralian common ancestor, this character will remain
307 a key aspect of understanding the spiralian developmental program, because its presence is highly
308 conserved across the group, while it origins and developmental contributions evolve in interesting
309 ways.

310 Whenever ectomesoderm evolved, it likely evolved in an organism with endomesoderm.
311 One question about the origin of ectomesoderm is whether it was a co-option or redeployment of
312 an endomesodemal gene regulatory network or the origin of a novel network. Available evidence

suggests that there is broad overlap in the gene regulatory networks between ectomesoderm and endomesoderm. For instance, a study in the gastropod *Crepidula fornicata*, surveyed the expression of developmental regulatory genes that have been implicated in mesoderm development in other animals (Perry et al. 2015). They found that eight conserved transcription factors were expressed in the endomesoderm, and of these, seven were also expressed in the ectomesoderm. In the ectomesoderm, they found expression of 12 genes, including 5 that were not shared with the endomesoderm[1]. While these similarities could have arisen by convergence, the simplest explanation is that some portion of the endomesoderm regulatory network was coopted to function in ectomesoderm, or that these were derived from a common ancestral type of mesoderm (Perry et al. 2015). From this perspective, it will be particularly interesting to identify the upstream components that initiate these two networks in spiralians, and perhaps other bilaterians.

If indeed these two sources of mesoderm do share substantial parts of their regulatory networks, then key questions become what aspects are different, and why? This is particularly interesting because they are generating very similar structures. For instance, both the ectomesoderm and endomesoderm are generating the larval retractor muscle, with no obvious qualitative difference in their roles, although endomesoderm contributes more. Similarly, their roles in head muscle are also very similar, despite that ectomesoderm has larger role. Genes like *ToSerpin1*, which are specific to ectomesoderm, might point to regulatory or functional

[1] The transcription factors *bra, cdx, foxA, gsc*, *otp, otx*, *six3/6* are expressed in both, *nk2.1 is only in endomesoderm, and snail*, *twist*, *hesA, hesB, notch2, are only in ectomesoderm (Perry et al, 2015). We did not include B-catenin in this list because its mRNA is ubiquitous.*

differences that are not clear from the lineage tracing of these populations. We note that the main functions of serpin family proteins are in post-translational regulation. Finding a serpin gene that plays a role in ectomesoderm development indicates that key regulatory events in this cell type may not be reflected in transcriptional differences. This suggests the possibility that post-translational regulation might have roles in the origin of evolutionary novelty.

Ablation of 3a and 3b mainly affects the anterior layer of radiating muscle, which is consistent with a role for 4d in the posterior but not anterior layer. However, the *ToSerpin1* MO knockdowns impact both layers, even though we have shown that the *ToSerpin1* expression is only in the 3a and 3b-derived cells. Thus, the *ToSerpin1* loss of function seems to have a stronger effect on the posterior layer than the complete ablation of the 3a and 3b cells. One possible explanation for this is that, in the 3a, 3b ablation (but not the *ToSerpin1* knockdown), the absence of the ectomesoderm cells causes 4d-derived cells to regulate, enabling them to better compensate for the absence of the 3a and 3b cells in the development of the posterior layer.

A more speculative, but intriguing explanation for why the MO has a stronger effect on the posterior layer than does the 3a, 3b ablation is that the *ToSerpin1*-MO 3a and 3b cells could be acting in a dominant negative fashion. In most animal systems that have been examined in detail, myogenesis involves fusion of muscle precursor cells. We wonder whether *ToSerpin1*-depleted cells might be interfering with the 4d cells' ability to make the posterior layer by failing in cell fusion events. This is one way that this apparently non-cell autonomous effect of the knockdown might be explained. We note that the retractor defects observed after 3a, 3b ablation are more severe than might be predicted from the relatively small contribution that 3a makes to this structure (Chan and Lambert 2011, 2014); this may also be a case of a non-autonomous effect in the interactions between ectomesoderm and endomesoderm.

Given the differences between spiralian endomesoderm and ectomesoderm, the latter clearly represents the evolution of a distinct cell type, at some phylogenetic level. Going further, since this population appears to have arisen as a novel gastrulation event, and that it can potentially make multiple types of cells, we argue that ectomesoderm belongs to the very small set of evolutionary novelties at the germ layer level of organization, including metazoan endomesoderm and the neural crest (Martindale and Henry 1999; Martindale et al. 2004; Osborne et al. 2018), it thus represents an important opportunity for comparative study of such events. The identification of *ToSerpin1*, and other ectomesoderm-specific factors should facilitate understanding the evolution of the ectomesoderm. We note that such factors may help identify putative ectomesoderm in spiralian embryos where cell lineage analysis remains challenging. This includes basal groups like gnathisomulids and rotifers, which will be crucial for understanding the origin of ectomesoderm and other possible spiralian innovations. In addition, analysis of orthologs of ectomesoderm-specific factors in non-spiralian metazoans could provide insight into the evolutionary origin of ectomesoderm.

Materials and Methods

Animal care and embryo collection

Tritia were collected near Woods Hole, MA, or ordered from the Marine Resources Center at the Marine Biological Labs in Woods Hole, MA. Embryos and larvae were collected and reared as previously described (Gharbiah et al. 2009a). The rearing temperature was 24 ° C (+/− 1).

Lineage tracing and cell ablation

Lineage tracing and cell ablation were adapted from previously described methods (Chan and Lambert 2011, 2014). Briefly, ten percent of CF568 dextran 10,000 MW, anionic and fixable (Biotium, CA, USA) was iontophoresed into cells using a current generator we built based on Hodor and Ettensohn, 1998. For 3a, 3b cell ablation, we filled the 3a and 3b cell until the cell lysed (Render, 1997). We checked 1 hour and 24 hours later to ensure the fluorescent dye was entirely gone, indicating successful ablation of the target cell lineages.

Transcriptome sequencing and differential gene expression analysis

RNA was extracted between 3a+48hrs and 3a+56hrs using NucleoSpin RNA XS (Macherey-Nagel, Düren, Germany). Then low input libraries for each sample (six libraries in total) were constructed, barcoded and then sequenced in one lane in a HiSeq 2500 by University of Rochester Genomics Research Center. Then we performed a *de novo* transcriptome assembly with Trinity (Haas et al. 2013) using default settings. We then used bowtie2 (Langmead and Salzberg 2012) to map reads to the assembly. After read mapping, we used two approaches for differential gene expressions. In the

first approach, we used RSEM (Li and Dewey 2011) to do read count and used Limma voom (Law et al. 2014) to call significant genes. 39 candidate genes from the significant genes were chosen from this analysis. These were chosen based on two criteria: 1) genes were down regulated 2) genes that had previously been shown to have regulatory roles, e.g. transcription factors. In the second approach, we first used a python script fasta2togff3.py (https://github.com/GMOD/Chado/blob/master/chado/bin/gmod_fasta2gff3.pl) to convert our transcriptome assembly to a gff file. Then we used HTseq (Anders et al. 2015) to do read counting, and DEseq (Love et al. 2014) to call significant genes. Nine candidate genes were chosen from this analysis for a total of 48 candidate genes. We designed primers for these 48 genes based on the sequences in the assembly. For 14 genes, either PCR amplification or probe synthesis failed, so we performed *in situ* hybridization for the remaining 34 out of 48 genes. For the seven genes that showed specific expression pattern, their GenBank accession numbers are: *ToSerpin1* (MT380182), *ToDachsous-like* (MT380183*), ToDigestive-gland-expressed-1* (MT380184), *ToApolipoprot-like* (MT380185), *ToUbiquitin-ligase-like* (MT380186), *ToMesoderm-expressed-1* (MT380187) and *ToProspero-like* (MT380188). For the other genes, their sequences are listed in Supplemental Table 1. We note that none of the ectomesoderm-expressed genes from Osborne et al. 2018 were found in our most differentially regulated genes from the RNA-seq experiment, or in our 48 candidate genes. This could be because they were not entirely specific to ectomesoderm, or perhaps because

411 the low-input RNA-seq we performed was not optimized to measure differences in low-copy
412 transcripts like those of transcription factors.

413

414 *In situ hybridization, phalloidin staining and imaging*

415 We performed *in situ* hybridization on these 34 genes with a mix of stages from early cleavage to
416 larval stages. *In situ* probe synthesis followed Kingsley et al. 2007 and *in situ* hybridization was
417 performed as previously described (Lambert and Nagy, 2002). Embryos were fixed in fresh Pipes-
418 EGTA-Magnesium (PEM) fixation buffer (10mM EGTA, 100mM PIPES (pH 6.9) and 1mM
419 $MgSO_4$) (Gharbiah et al. 2009b), and stained with Phalloidin Alexa Fluor 488 (Molecular Probes,
420 OR, USA) overnight and then washed 3 times with PBTw before being stored in mount (80%
421 glycerol; 1x PBS) for imaging. *In situ* hybridization was imaged on a Zeiss Axioplan 2 microscope.
422 The imaging of lineage tracing and Phalloidin actin staining was performed on a Leica SP5 confocal
423 microscope. The Z-projections of the confocal stacks were calculated by standard deviation method
424 in ImageJ (Abràmoff et al. 2004).

425

426 *MO design and injection*

427 *ToSerpin1* MO1 was designed by the vendor (Gene Tools, OR, USA). The sequence of *ToSerpin1*
428 MO1 and MO2 are 5'ATGCCAGCCGAAAATCTATCGCAGT 3' and 5'
429 ACGCAAAACTATTTTCGACTTTGCA 3'. The sequence of control MO is
430 5' TTCAGTCCATGTCAGTGTCCAAGCC 3'. Zygote injection was performed as described
431 previously (Rabinowitz et al. 2008), and injected animals were reared until they depleted their yolk
432 for scoring. The indicated concentrations for injected solutions are the concentrations in the pipette.

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Table 1: The phenotypes of ablation and MO injection.

	Velar lobes normal size	Anterior ring muscle present	Posterior ring muscle present	Anterior ring muscle normal	Posterior ring muscle normal	Normal amount of radiating muscle	Anterior radiating muscles complete (1)	Posterior radiating muscles complete (1)
Control MO 0.75 mM	24/24	24/24	24/24	24/24	24/24	24/24	11/12	11/12
3a, 3b ablation	0/18	1/18 (2)	1/18 (2)	0/18	0/18	0/18	0/9	9/9
ToSerpin MO1 0.75 mM	2/40	35/40	19/40	15/40	10/40	19/40	4/20	8/20
MO1 with Serpin mRNA-5' UTR ablation (3)	36/40	40/40	38/40	34/40	31/40	38/40	19/20	17/20
ToSerpin MO2 0.85 mM (4)	15/26	26/26	8/26	8/26	5/26	22/26	8/13	2/13

(1) For both of these columns we only scored one velar lobe per animal because it was generally not possible to successfully orient both velar lobes in one larva for confocal microscopy.
(2) In the cases where an anterior and posterior ring muscles were present, they were incomplete; the partial ring muscles were in different larvae.
(3) The MO was injected at 0.75mM and the mRNA was injected at 150 ng/ul.
(4) MO2 had a similar dose dependency to MO1, with no effects around 0.5 mM, and lethality during organogenesis around 1 mM. For this MO the concentration with the strongest effect on velar muscle morphology but essentially no lethality was at 0.85 mM, slightly higher than MO1.

Figures and legends

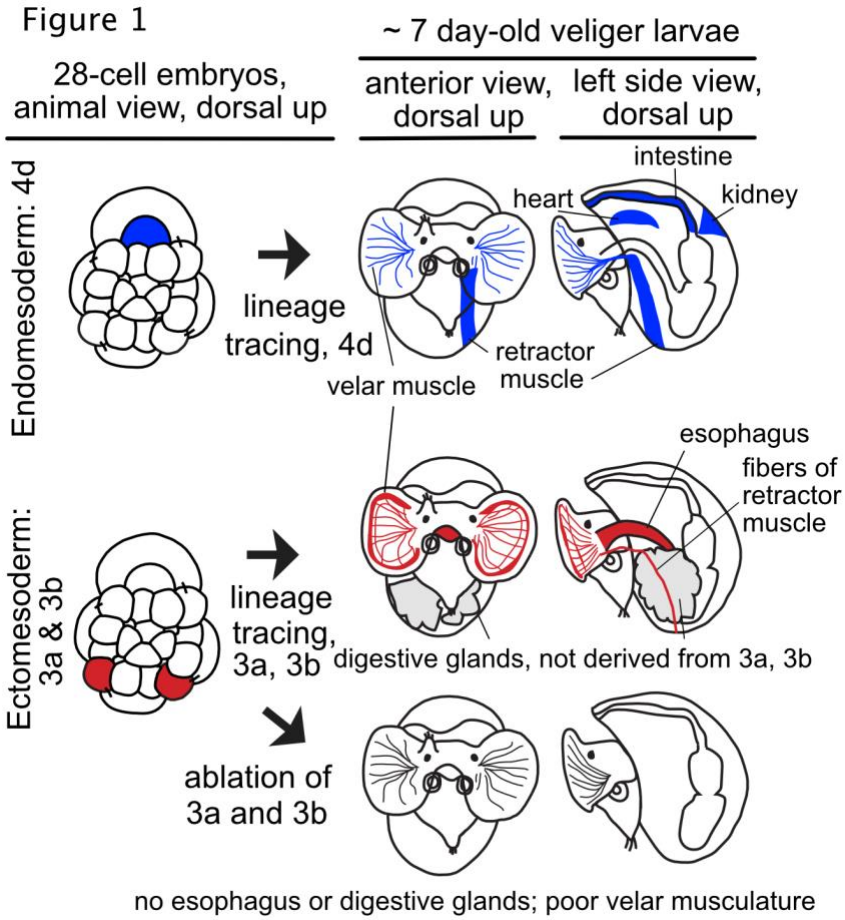

Figure 1: Mesoderm comes from two sources in the snail *Tritia*, and both sources contribute to the same muscles in the larva. The cell 4d (blue) generates endomesoderm: the intestine, and most of the mesoderm in the larva, including the heart, kidney, main larval retractor muscle, and some of the muscle in the velar lobes of the head. The other source of mesoderm is ectomesoderm, which mainly derives from the cells 3a and 3b, but also includes smaller contributions from 2b and 2c (see text for details). 3a and 3b (red) generate most of the ectomesodermal muscle in the head (velar muscle), and make a small contribution to the main larval retractor. These cells also make the esophagus. Ablating both 3a and 3b results in the loss of the esophagus and much of the head musculature. These animals also generally lack the digestive glands, even though these cells do not contribute to them, and often have defects in the larval retractor (Chan and Lambert, 2014; this study).

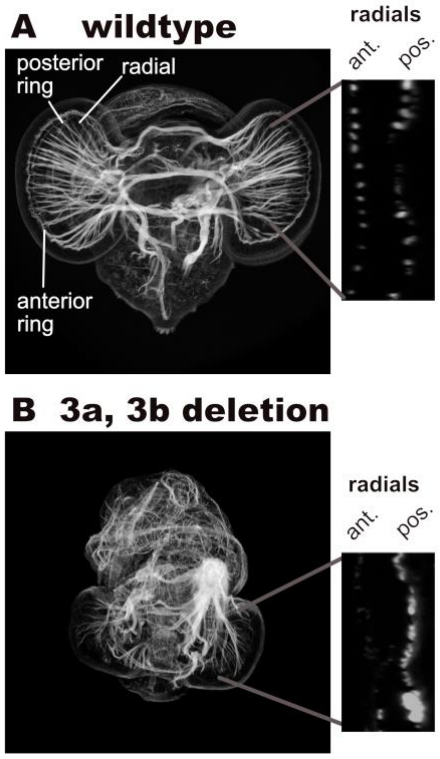

Figure 2: The muscle system in the velar lobe, and the results of 3a, 3b ablation. A) Anterior view of a control veliger, around 7 day-old (dorsal up, ventral down). Muscles are stained with phalloidin (white). The anterior and posterior ring muscles are visible, as are the radiating muscles that run from the center of the head out to the ring muscles. The panel to the right shows a confocal cross section of a control left velar lobe, at approximately the location indicated by the lines. There are two layers of radiating muscles, the anterior is closer to the surface of the velum (towards the viewer in A), and posterior is behind that (from the perspective of the viewer in A). B) Anterior-dorsal view of a larva after ablation of 3a and 3b (Because these animals are somewhat misshapen we cannot mount them in a full anterior view like the control). Both ring muscles are absent, and there are fewer radiating muscles. The panel on the right shows a cross section of a 3a, 3b ablation velar lobe, at approximately the location indicated by the lines. The anterior layer is mostly gone, and the posterior layer is present, but is disorganized compared to control.

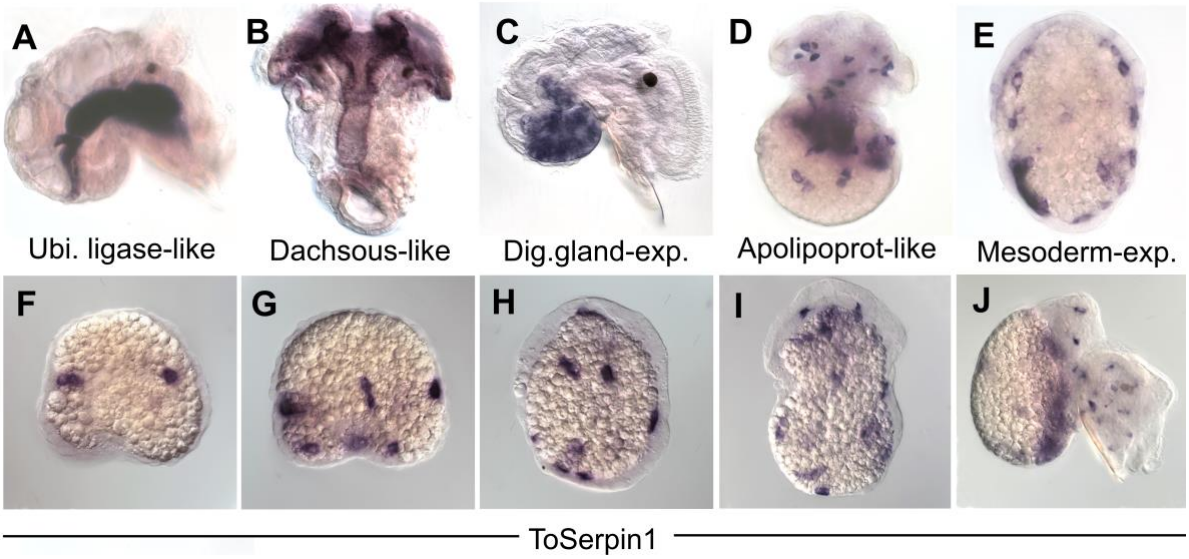

Figure 3: *In situ* hybridization showing expression of transcripts whose levels go down after ablation of 3a and 3b. Top row (A-E): Transcripts with specific patterns consistent with 3a, 3b ablation, that were not further characterized. (A-C are in hatchling-stage larva, around 7 days after egg laying. A and C are right side views, B is dorsal with anterior up.) A) *ToUbiquitin-ligase-like* is specific to the esophagus and the anterior wall of the stomach. B) *ToDachsous-like* is expressed in the esophagus and the velar lobes. C) *ToDigestive-gland-expressed-1* is expressed in the digestive glands, which are lost after 3a, 3b ablation. D) *ToApolipoprotein-like* is expressed in scattered mesodermal cells that appear to be ectomesoderm, as well as an unidentified region in the center of the embryo. This is a 5 day-old larva, dorsal view, anterior up. E) *ToMesoderm-expressed-1* is a gene with only weak homology outside of molluscs and no predicted function. It is expressed in scattered internal cells that appear to be ectomesoderm, as well as other internal cells that are probably endomesodermal, especially in the left posterior (compare to I). This is a three-day-old embryo, dorsal view, anterior is up. Bottom row, F-J, shows a developmental series of expression for *ToSerpin1*, the focus of this report. F) In a 48-hour embryo, at the end of gastrulation, *ToSerpin1* is expressed in 2-3 internal cells; this embryo has a cluster of two on the left side and one on the right side (dorsal view, anterior up). G) In an embryo at ca. 60 hours, there are 8-10 *ToSerpin1*-positive cells (dorsal view, anterior up). During organogenesis, at around 3 days (H) and 4 days (I), there are increasingly more scattered internal cells expressing *ToSerpin1* (dorsal views, anterior up). J) In an early veliger (ca. 6 days), *ToSerpin1* cells are present in the head, foot and body (right side view).

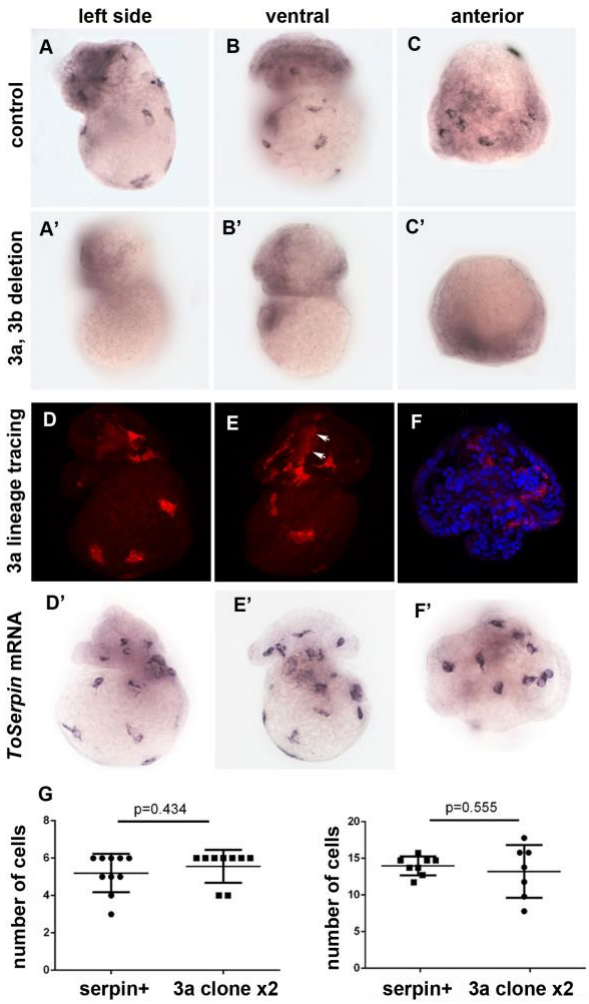

Figure 4: *ToSerpin1* is specific to ectomesoderm. A-C') Internal *ToSerpin1* cell stained by *in situ* hybridization in control embryos (A-C) are not observed in embryos after 3a, 3b ablation (A'-C'). These larvae are synchronously developing siblings from one egg capsule. The diffuse staining remaining in (A'-C') is apparently a result of extended culture outside of the egg capsule that is necessitated by the ablation protocol; we were unable to remove it, and did not observe it with the same probe on embryos that were fixed shortly after being removed from egg capsule, e.g. Figure 3, F-J, so we do not think it is a component of the normal *ToSerpin1* expression pattern, which is the scattered distinct cells in A-C. (D-F') 3a lineage tracing (D-F; red stain) reveals ectomesodermal cells in head and posterior. *In situ* staining for *ToSerpin1* in synchronously developing sibling embryos is shown in D'-F'. Note that the 3a traced and *ToSerpin1* positive cells are morphological similar. The non-ectomesoderm cell of 3a and 3b cell lineages (non-muscle cells), such as the tube-like esophagus in the midline of the larva (indicated with white arrows in E) and cells in the edge of the velum are excluded from the analysis. These cells are spatially and morphologically distinct from ectomesoderm cells. G) Counts of *ToSerpin1*-positive cells, versus counts of 3a labeled cells, which are doubled to account for the bilaterally symmetrical 3b contribution to ectomesoderm population (Chan and Lambert 2014). Anterior head region is on the left, posterior region is on the right. In both regions, there is no significant difference between the number of ectomesoderm cells and *ToSerpin1*-positive cells.

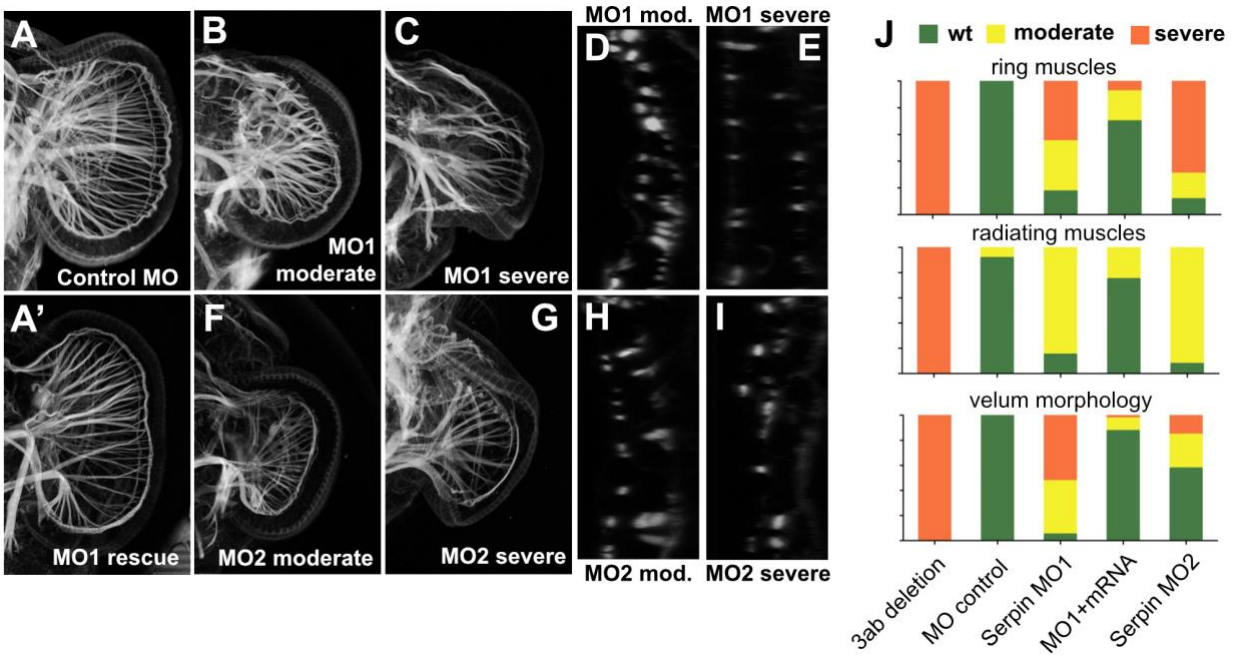

Figure 5: *ToSerpin1* knockdown disrupts velar muscle development. A-C, A', F, G are anterior views of left velar lobes, with muscle stained with phalloidin. D, E, H, and I are confocal cross sections, as in Figure 2, with anterior up. A) After injection of a control morpholino at 0.75 mM, velar morphology is wildtype. A') A *ToSerpin1* mRNA without the MO1 binding site, injected along with the MO, largely rescues velar development (compare to B and C). Injection of *ToSerpin1*MO1 disrupts velar musculature; (B) shows a moderate example with a smaller lobe and disorganized radiating muscles, while (C) shows a severe example with a smaller lobe, fewer radiating muscles and only the anterior ring muscle which is poorly developed. Cross sections (D, E) show defects in both the anterior and posterior radiating layers. Injection of the non-overlapping MO2 has similar effects (F-I). J) Comparison of velar muscle phenotypes after various treatments. Embryos were considered "wildtype" if no abnormality was detected; if any structure was missing, embryos were assigned into the "severe" category; all other embryos were assigned into the "moderate" category.

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