The *C. elegans* embryonic fate specification factor EGL-18 (GATA) is reutilized downstream of Wnt signaling to maintain a population of larval progenitor cells

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In metazoans, stem cells in developing and adult tissues can divide asymmetrically to give rise to a daughter that differentiates and a daughter that retains the progenitor fate. Although the short-lived nematode *C. elegans* does not possess adult somatic stem cells, the lateral hypodermal seam cells behave in a similar manner: they divide once per larval stage to generate an anterior daughter that adopts a non-dividing differentiated fate and a posterior daughter that retains the seam fate and the ability to divide further. Wnt signaling pathway is known to regulate the asymmetry of these divisions and maintain the progenitor cell fate in one daughter, but how activation of the Wnt pathway accomplished this was unknown. We describe here our recent work that identified the GATA transcription factor EGL-18 as a downstream target of Wnt signaling necessary for maintenance of a progenitor population of larval seam cells. EGL-18 was previously shown to act in the initial specification of the seam cells in the embryo. Thus the acquisition of a Wnt-responsive *cis*-regulatory module allows an embryonic fate specification factor to be reutilized later in life downstream of a different regulator (Wnt signaling) to maintain a progenitor cell population. These results support the use of seam cell development in *C. elegans* as a simple model system for studying stem and progenitor cell biology.

β -Catenin Dependent Wnt Signaling in *C. elegans*

The β -catenin dependent Wnt signaling pathway is one of a core set of extracellular signaling pathways that are used repeatedly throughout the course of metazoan development.¹⁻³ The nematode *C. elegans* employs Wnt signaling throughout its development, however in this model organism there are 2 β -catenin dependent Wnt signaling pathways. The *C. elegans* Wnt/BAR-1 canonical (WBC) and Wnt/ β -catenin asymmetry (WBA) pathways both culminate in β -catenin dependent transcriptional regulation of target genes, however the manner by which pathway activation accomplishes this differs.^{4,5} The WBC pathway is activated in the same manner as the 'canonical' Wnt signaling pathway in vertebrates; in the absence of Wnt signal the transcription factor β -catenin is subjected to proteasomal degradation, but in the presence of ligand β -catenin is stabilized and forms a complex with a TCF family member to activate target gene expression. The WBA pathway is a nematode specific variant that regulates many asymmetric divisions in *C. elegans* and its hallmark is the asymmetric distribution of Wnt pathway components in the mother cell and daughter cells of an asymmetric division. In such divisions, in the daughter cell displaying Wnt pathway activation, a kinase cascade causes export of the TCF homolog POP-1 from the nucleus, lowering its level to facilitate formation of a complex with β -catenin which activates target gene

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Abbreviations: WBC, Wnt/BAR-1 canonical; WBA, Wnt/ β -catenin asymmetry; VPC, vulval precursor cell

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Figure 1. Seam cell division pattern. (A) Diagram of a newly hatched L1 hermaphrodite larva showing the position of 10 seam cells along the lateral midline on the left side (anterior is left, dorsal is up). (B) Diagram showing the division pattern of a representative 'V' class seam cell. The seam cell is specified in the embryo but does not divide. During each larval stage the seam cell divides once along the anterior-posterior axis to generate an anterior daughter that differentiates (usually as a hypodermal cell 'H' that joins the syncytial hypodermis) and a posterior daughter that retains the seam cell fate and the ability to divide further (blue lineage). In the early L2 stage, the seam cell divides once in a symmetric manner to generate 2 seam daughters that subsequently only divide asymmetrically. After their division in the L4 stage, the seam cells themselves exit the cell cycle and fuse homotypically to form a long syncytial seam cell that secretes the adult cuticle alae (blue lines).

expression, causing the Wnt signaled daughter cell to adopt a specific fate. The non-Wnt responding daughter cell continues to have high nuclear POP-1 levels, repressing target gene expression and driving adoption of a fate different from its sister.

Wnt Signaling and the Stem Cell-like Division of the Hypodermal Seam Cells

One process regulated by the WBA pathway is the asymmetric division of the hypodermal seam cells during larval development. The hypodermis of the worm is the single cell layer thick skin, which serves both structural and protective functions.^{6,7} For example, the hypodermal cells secrete the cuticle, the collagen-rich outer layer of the worm that is resynthesized before each molt.^{7,8} The seam cells are specialized hypodermal cells arrayed in

a single row on both sides of the worm from anterior to the posterior⁶ (Fig. 1A). Ten seam cells per side are born and specified during embryogenesis but do not divide. Then, during each of the 4 larval stages most seam cells divide in an asymmetric manner to generate a posterior daughter that retains the seam cell fate and the ability to divide, and an anterior daughter that terminally differentiates, in most cases generating a hypodermal cell that fuses with the surrounding skin syncytium^{6,9} (Fig. 1B). These reiterated asymmetric stem cell-like divisions allow seam cell self renewal while contributing numerous cells to the growing skin of the animal during larval life. After their final division in the mid L4 stage all of the seam cells exit the cell cycle, terminally differentiate, and fuse to form a single long cell that secretes a specialized cuticular structure called the adult alae (Fig. 1B). Wnt signaling was first identified to regulate fate specification during

the division of individual seam cells; subsequently the WBA pathway was shown to function in the asymmetric divisions of most seam cells during larval life.¹⁰⁻¹³ For example, we showed that reduction of Wnt pathway activity causes the posterior daughter to adopt the fate of its anterior hypodermal sister thus decreasing terminal seam cell number, while over activation of the Wnt pathway causes both daughters of a seam cell division to adopt the seam fate resulting in too many seam cells¹¹ (Figs. 2B, 2C). This and earlier results therefore indicate that Wnt signaling is not necessary for seam cell division per se, but is required for the asymmetric nature of the daughter cell fates.

Although the short lived *C. elegans* does not possess adult somatic stem cells, the behavior of the seam cells during larval development is reminiscent of the behavior of progenitor cells in adult tissues of vertebrates. For example, during larval life, the seam cells are committed to the hypodermal fate but have not terminally differentiated, they have the ability to divide multiple times to generate differentiated hypodermal and neuronal cell types, and during wild type development they exit the cell cycle and terminally differentiate after a finite number of cell divisions.⁹ Further, Wnt pathway signaling is responsible for the maintenance of the progenitor-like cell fate during the asymmetric divisions of the seam cells, and Wnt signaling is known to regulate the behavior of progenitor cells in vertebrate tissues.^{14,15} Therefore understanding the genetic architecture regulating the asymmetric cell division of the *C. elegans* larval seam cells could shed light on questions fundamental to metazoan development and homeostasis.⁹ Knowing this, a key question becomes what target genes are activated by Wnt signaling to allow the *C. elegans* larval seam cells to behave in a manner similar to stem and progenitor cells in adult somatic tissues of larger metazoans?

Identifying Wnt Pathway Target Genes in *C. elegans*

Compared to our knowledge in vertebrates, very little is known about Wnt pathway target genes in *C. elegans*.^{16,17} Several approaches can be taken to address

the question of target gene identity. For example, molecular and genetic techniques can identify Wnt target genes that give a phenotype similar to Wnt pathway mutants for a given process; this is the manner in which most known *C. elegans* Wnt pathway targets were identified.^{18–26} Knowing the preferred binding site of a transcription factor allows use of bioinformatics approaches to identify possible target genes. However, since the POP-1 consensus binding site is predicted to be present roughly once per kilobase in the genome,¹⁶ the near ubiquity of POP-1/TCF binding sites in the *C. elegans* genome makes *de novo* bioinformatics approaches difficult, however this approach has recently been used to identify Wnt regulated genes.²⁷ Chromatin immunoprecipitation efforts with POP-1 have also been hampered by the ubiquity of binding sites and by sickness caused by transgenic arrays carrying GFP-tagged POP-1 (K. Thompson and D. Eisenmann, unpublished results). As an alternative approach we previously used conditional activation of Wnt signaling and microarray analysis to identify transcripts upregulated in response to Wnt pathway activation *in vivo*.¹⁶ In this technique, the heat shock promoter was used to express a stabilized gain-of-function variant of the *C. elegans* β -catenin BAR-1 that can cause a Wnt gain-of-function phenotype in both vulval development and seam cell division.^{11,28} Jackson et al subjected synchronized populations of animals to a heat shock at a defined time in larval life and compared transcription profiles from worms subjected to Wnt pathway activation to those given only the heat shock and used microarray analysis to identify over 100 genes upregulated upon Wnt signaling.¹⁶ After further validation *in vivo*, 14 genes were shown to be *bona fide* Wnt target genes, although whether they are direct targets could not be determined given the caveats discussed above.

Figure 2. Wnt signaling regulates the asymmetry of the larval seam cell divisions. (A) In wild type animals, each seam cell divides once per larval stage to generate anterior hypodermal daughter ('hyp') that differentiates and a posterior daughter that maintains the seam cell fate ('SC') and the ability to divide further (vertical arrow). (B) When Wnt signaling pathway activity is reduced, seam cells are seen to divide symmetrically to generate 2 daughters that adopt the anterior 'differentiated cell' fate. (C) When Wnt signaling pathway activity is increased, seam cells are seen to divide symmetrically to generate 2 daughters that adopt the posterior 'progenitor cell' fate.

Several of these gene encoded cuticle collagens normally expressed in the mid L4, indicating a previously unknown role for Wnt signaling in regulating adult cuticle development.¹⁶

Identifying Wnt Pathway Target Genes in the Larval Seam Cells

The analysis of Jackson et al showed that the conditional activation/microarray method could identify previously unknown Wnt pathway targets, however few of the genes found played an obvious role in development of the vulval precursor cells (VPCs) or seam cells, the 2 larval cells types we had shown were dependent on Wnt signaling for proper fate specification. One reason may be that we examined gene expression from whole animals, and identified genes expressed in the most cells or expressed at the highest levels. However, the cells we are interested in make up a tiny fraction of the cells in a larval worm: there are 32 seam cells and 6 VPCs in an early L3 stage worm, out of > 1000 somatic and germ cells.²⁹ One approach that has been used successfully to catalog gene expression in small numbers of embryonic cells is the dissociation of embryos expressing GFP in a defined cell type, followed by sorting and the use of GFP positive cells for gene expression analysis.^{30,31} Unfortunately the thick

cuticle of the larval worm is more difficult to dissociate than the embryonic eggshell, and the cells we are interested in are physically attached to the cuticle, rendering this technique unattractive.

To identify Wnt pathway targets specifically in the larval seam cells we combined our previous conditional Wnt activation approach with a cell type-specific profiling technique called 'mRNA tagging'.³² In this method an epitope-tagged polyA binding protein is expressed in the cell type of interest, proteins and mRNA are crosslinked *in vivo*, then transcripts from the cells of interest are isolated following affinity purification of the tagged polyA binding protein and cross-link reversal. This method has been successfully used to characterize the larval transcriptomes of muscle and neuronal cell types.^{31–35} We believe we are the first to combine this method with the conditional activation of an extracellular signaling pathway, as described below.

In our recent work, we expressed the tagged polyA binding protein using an enhancer active in the larval seam cells and VPCs³⁶ (Gorrepalli et al submitted). We conditionally activated the Wnt signaling pathway as before using heat shock expression of the amino-terminally deleted BAR-1 protein at the L2/L3 molt. We also conditionally inhibited Wnt signaling in other animals using heat shock expression of a dominant negative POP-1/TCF

variant that lacks the β catenin binding domain; expression of this protein leads to Wnt reduction of function phenotypes in several cell types.^{11,37} Animals in which Wnt signaling was activated and inhibited along with control heat shock animals were subjected to affinity purification to isolate mRNA pools enriched for transcripts from the seam cells and VPCs and these samples were subjected to microarray analysis to examine gene expression profiles. By comparing transcript levels in Wnt activated versus Wnt inhibited populations, we identified 239 putative Wnt pathway targets differentially expressed in the seam cells and VPCs in response to Wnt signaling (Gorrepati et al submitted). Interestingly, we identified the GATA factor encoding genes *egl-18* and *elt-6* as putative targets of Wnt signaling.³⁶ These factors seemed like prime candidates to be effectors of Wnt signaling that regulate fate in the larval asymmetric seam cell divisions because of their known role in initial embryonic specification of the seam cells.

Specification of the Embryonic Seam Cells Requires GATA Factors ELT-1, EGL-18 and ELT-6

During embryogenesis, a GATA transcription factor network comprising ELT-1, EGL-18 and ELT-6 is required for the initial specification of the seam cell fate in 10 cells per side.⁶ While *elt-1* is required to generate all epidermal cells (seam cells, P cells and syncytial hypodermal cells),^{38,39} *egl-18* and *elt-6* are specifically required for specifying seam cell fate.⁴⁰ *egl-18* and *elt-6* are adjacent genes that encode related GATA type transcription factors that are expressed in the embryonic cells fated to become seam cells.⁴⁰ Loss of function of one or both factors by RNAi causes loss of expression of several seam specific genes, ectopic expression of *elt-3*, a GATA factor normally expresses in non-seam hypodermal cells, and fusion of the seam-fated cells with the surrounding hypodermis, leading to fewer seam cells in early larval worms. These results led to the model that these GATA factors are required downstream of ELT-1 to specify the seam cell fate initially in the embryo,

and may also act in maintenance of the seam cell fate in larval life.⁴⁰ However, it was not clear how these factors may be regulated during larval life to perform their proposed functions. In addition, there was no evidence for the function of Wnt signaling in embryonic seam cell specification, so the identification of these genes as potential targets downstream of Wnt in the larval seam cells was a novel result.

***egl-18* and *elt-6* Act Downstream of Wnt Signaling to Maintain the Seam Cell Fate During Larval Asymmetric Cell Divisions**

Consistent with a function in larval seam cell asymmetric divisions, we found that an *egl-18* transcriptional reporter is expressed strongly in the posterior daughter of seam cell divisions that adopts the seam fate, while fading in the anterior daughter that differentiates.³⁶ Further, reduction of *egl-18* and *elt-6* function only in larval animals (after embryogenesis is complete) led to a decrease in the number of seam cells in young adults. Consistent with a role downstream of Wnt signaling, we showed that *egl-18* reporter expression is increased or decreased after Wnt pathway activation or inhibition respectively, and that *egl-18* activity is necessary for the hypodermal to seam cell fate transformations induced by Wnt pathway overactivation. A single POP-1/TCF binding site in an upstream region of *egl-18* was required for strong expression of the *egl-18* reporter in the larval seam cells, and POP-1 bound to this site *in vitro*. Finally, over expression of EGL-18 was sufficient to drive the expression of a seam cell marker in other hypodermal cells in wild type larvae, and in anterior hypodermal-fated daughters in a Wnt pathway-sensitized background, suggesting that *egl-18* expression may be instructive and not just permissive for seam cell fate specification. Based on these results, we proposed that *egl-18* (and *elt-6*) are reiteratively activated by Wnt signaling during the larval asymmetric seam cell divisions to facilitate the specification of seam cell fate in only one of the daughters of each division thereby allowing the

renewal of this progenitor cell population after each cell division³⁶ (Fig. 3).

The Reutilization of Embryonic Specification Factors in Postembryonic Development

These data suggested that 2 GATA factors required for seam cell specification in the embryo independent of Wnt signaling, are reused downstream of Wnt signaling during larval life. During metazoan development, it is not uncommon for transcription factors to be re-used during larval or adult life to recapitulate aspects of embryonic development of a specific cell type or tissue. For instance, recent work has shown that the transcriptional cascade comprising Pax6, Neurog1 and Tbr1 and 2 used during embryonic neurogenesis is utilized again during adult neurogenesis.⁴¹ Similarly, during mammalian skeletal muscle development, transcription factors Pax3 and Pax7 not only function during myogenesis in the embryo but are also crucial for maintaining the adult skeletal muscle stem cell population and initiating myogenesis in response to injury and muscle wasting.⁴² In *C. elegans*, the Hox gene *lin-39* is required in early larval life to specify a set of ventral hypodermal blast cells called the Vulval Precursor Cells.^{43,44} Later in larval life Wnt signaling is required for these cells to maintain expression of *lin-39* and their VPC fate,²⁴ however Wnt signaling is not required in the earlier *lin-39*-dependent fate specification process, and some factors required for initial embryonic expression of *lin-39* in the precursors to these cells do not have a role in later larval expression.⁴⁵ The question that arises is why do seam cells re-use these GATA factors in larval life?

In the *C. elegans* embryo, the fate choices of embryonic cells are controlled by the asymmetric segregation of transcription factors and the asymmetric activation of signaling pathways at early cell divisions.⁴⁶ Such processes lead to expression of the transcription factor ELT-1 in the descendants of the founder cell AB that will become the embryonic seam cells, causing them to adopt a general epidermal fate, however the exact factors or signals required for early ELT-1

Figure 3. EGL-18 mediates seam cell fate specification in the embryo and larva downstream of different inputs. (A) During embryogenesis, cells in the AB lineage that are fated to become seam cells are first specified to adopt an epidermal fate ('epi') via action of the GATA factor ELT-1. Later, expression of CEH-16 in these cells leads to expression of the GATA factor EGL-18 (these cells also express the related GATA factor ELT-6, not shown), leading them to adopt the lateral hypodermal or seam cell fate ('SC'). These cells do not divide until larval life begins. (B) In larval life, some seam cells divide asymmetrically to generate a posterior daughter that retains the seam cell fate and the ability to divide further. Maintenance of this progenitor cell fate requires activation of the Wnt signaling pathway to maintain expression of *egl-18* in only that daughter cell of the division. Thus an embryonic fate specification factor is reutilized to maintain a progenitor cell population in larval life, and *egl-18* specifies the seam cell fate both embryonically and post-embryonically, but in response to different input signals.

expression in the prospective seam cells are unknown⁶ (Fig. 3A). Further unknown events lead to expression of the Engrailed homolog CEH-16 in these cells, which is necessary for expression of EGL-18 and ELT-6 and adoption of the seam cell fate.^{40,47} It is parsimonious to extend this basic mechanism to maintain seam cell identity in larval life as well: there is no need to 'reinvent the wheel'. However, once embryogenesis is complete, this unique combination of factors and signals is unlikely to be present in the larva. Further, the larval seam cells undergo asymmetric divisions that require the action of the fate specification factors to be restricted to only one daughter of a division (Fig. 3B). Given this, it is not unreasonable that the regulation of *egl-18* and

elt-6 should come under the WBA pathway that controls many asymmetric divisions in *C. elegans*,⁵ perhaps through acquisition of POP-1 binding site in the upstream region.³⁶ In this way, these master regulators of seam cell fate that are initially expressed in response to one set of factors in the embryo can be reutilized downstream of Wnt signaling during asymmetric larval cell divisions to maintain the progenitor seam fate in one daughter.

A number of questions remain about the role of Wnt signaling and the GATA factors EGL-18 and ELT-6 in the development and division of the seam cells. For example, it is still not clear what factors lead to the asymmetric activation of the Wnt pathway in just the posterior

daughter of each larval seam cell division, when the seam cells themselves are arrayed along the anterior-posterior axis.^{10,11} Also, it will be interesting to identify the targets of EGL-18 and ELT-6 that allow them to execute and maintain the progenitor-like seam cell fate. *egl-18* activity is required for expression of several nuclear hormone receptors in the seam cells, and for repression of the GATA factor ELT-3 and the fusogen EFF-1.^{40,47} Other factors identified in the conditional Wnt activation array experiments may also function downstream of EGL-18 and ELT-6 (Gorepati et al, submitted). Considering the crucial role Wnt signaling and GATA factors play in regulating stem cell biology in many mammalian tissues, the *C. elegans* seam cells should provide an excellent model to elaborate the relationships between these important factors in metazoan development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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