The TGF- β Family in *Caenorhabditis elegans*

Cathy Savage-Dunn¹ and Richard W. Padgett²

¹Department of Biology, Queens College, and the Graduate Center, New York, New York 11367

²Waksman Institute, Department of Molecular Biology and Biochemistry, Cancer Institute of New Jersey, Rutgers University, Piscataway, New Jersey 08854-8020

Correspondence: cathy.savedunn@qc.cuny.edu; padgett@waksman.rutgers.edu

Transforming growth factor β (TGF- β) and related ligands have potent effects on an enormous diversity of biological functions in all animals examined. Because of the strong conservation of TGF- β family ligand functions and signaling mechanisms, studies from multiple animal systems have yielded complementary and synergistic insights. In the nematode *Caenorhabditis elegans*, early studies were instrumental in the elucidation of TGF- β family signaling mechanisms. Current studies in *C. elegans* continue to identify new functions for the TGF- β family in this organism as well as new conserved mechanisms of regulation.

The transforming growth factor β (TGF- β) family is one of the major signal transduction pathways used in animals for development and homeostasis. As might be expected of potent growth factors, misregulation or mutation in family signaling components contributes to a variety of diseases and oncogenic growth. TGF- β appears to be an animal invention, being found in the most primitive animals, sponges and Trichoplax (Srivastava et al. 2008, 2010; Huminiecki et al. 2009), but not in yeast or plants. Since its discovery more than 35 years ago (de Larco and Todaro 1978; Moses et al. 1981; Roberts et al. 1981), 33 genes encoding TGF- β -related proteins have been identified in humans, seven in *Drosophila* and five in *Caenorhabditis elegans* (Table 1). Members of this family include TGF- β , activins, bone morphogenetic proteins (BMPs), nodal, various growth and differentiation factors (GDFs), and Müllerian inhibiting substance. The li-

gands are expressed as longer precursors that undergo proteolytic cleavage, resulting in disulfide-linked homodimers or heterodimers that bind specific cellular receptors. In some cases, ligands are generated as latent complexes that later become activated.

Signaling is initiated with ligand binding to two related serine–threonine kinase transmembrane receptors (type I and type II), which suggests an ancient duplication of the genes encoding the receptors. The discovery of serine–threonine kinase receptors in animals suggested that the downstream signaling components might be different from known signal transducers for tyrosine kinase receptors, which turned out to be true. TGF- β family receptors exist as tetramers containing two type I and two type II receptors. The receptors can phosphorylate not only serine and threonine residues but tyrosine residues as well (Lawler et al. 1997). Binding of ligand to the receptors causes the type II recep-

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Table 1. List of transforming growth factor β (TGF- β) family signaling components in *Caenorhabditis elegans*

Function	Gene name
Ligands	<i>dbl-1</i>
	<i>daf-7</i>
	<i>unc-129</i>
	<i>tig-2</i>
	<i>tig-3</i>
Type I receptors	<i>sma-6</i>
Type II receptor	<i>daf-1</i>
	<i>daf-4</i>
R-Smads	<i>sma-2</i>
	<i>sma-3</i>
	<i>daf-8</i>
	<i>daf-14</i>
Co-Smads	<i>sma-4</i>
	<i>daf-3</i>
I-Smad	<i>tag-68</i>
SnoN/Ski	<i>daf-5</i>
Schnurri	<i>sma-9</i>

tor to phosphorylate the GS domain on the type I receptor, thereby activating its kinase activity (Shi and Massagué 2003). Receptor-regulated Smads (R-Smads) are recruited and phosphorylated at their carboxyl termini, which then bind to a co-Smad, and accumulate in the nucleus.

Evolutionary studies have shown that the most primitive animals, such as sponges and Trichoplax, have four classes of Smads: two R-Smads, one co-Smad, and one inhibitory Smad (I-Smad). One R-Smad clade transmits BMP signals, whereas the other R-Smad clade is a non-BMP signaling group, commonly referred to as the TGF- β /activin Smads (Huminięcki et al. 2009). Smads contain two conserved domains, the amino-terminal MH1 (Mad-homology 1) and carboxy-terminal MH2 (Mad-homology 2) domains, separated by an unstructured linker region (Macias et al. 2015). The MH1 domain binds directly to DNA (Shi et al. 1998). A conserved lysine-rich motif in the MH1 domain likely acts as a nuclear localization signal. The MH2 domain mediates protein-protein interactions, including trimerization in the Smad complex (Chacko et al. 2004). Within the MH2 domain of R-Smads, the L3 loop me-

diates contact with the type I receptor. Specific sequence signatures in the L3 loop discriminate between BMP Smads (Smad1 and Smad5 in vertebrates) and non-BMP (TGF- β /activin) Smads (Smad2 and 3 in vertebrates) (Lo et al. 1998). Smads bind regulatory sites in DNA only weakly, so they require association with other transcription factors to increase affinity for DNA, and to cooperatively direct transcription of downstream targets. In addition to signaling through Smad pathways, output from TGF- β family receptors also occurs through noncanonical pathways without Smad involvement, including activation of ERK, JNK, and p38 MAPK, and Akt signaling.

Regulating the pathway is important, given the wide range of developmental processes in which TGF- β family members participate, particularly because many ligands have overlapping expression patterns. Further complicating matters is the fact that the ligands encoded by 33 genes signal through only five R-Smads (two clades) and one common co-Smad in vertebrates. Specificity is achieved by several different mechanisms, with many others likely to be discovered. Phosphorylation and dephosphorylation of signaling components, including receptors and Smads, frequently control signaling intensity. Other modifications of TGF- β signaling components include N-glycosylation, ubiquitylation, sumoylation, neddylation, and methylation.

DISCOVERY OF THE TGF- β FAMILY PATHWAY IN *C. elegans*

The first TGF- β family components identified in *C. elegans* were in a dauer pathway regulating dauer development. The dauer form is an alternative third larval stage, in which the nematode suspends development for potentially long periods of time due to harsh environmental conditions (Riddle and Albert 1997). Mutations in TGF- β family components of the dauer pathway result in a dauer-constitutive phenotype, in which nematodes arrest development under normal environmental conditions. One of the first of these genes cloned was *daf-1*, which encodes the first identified TGF- β family type I

receptor (Fig. 1) (Georgi et al. 1990). However, because the dauer pathway was not known to be controlled by a TGF- β family protein at the time, DAF-1 was not recognized as the first TGF- β family receptor, but rather viewed as an orphan receptor. Once the activin type II receptor was cloned (Mathews and Vale 1991), DAF-1 was recognized as a TGF- β family receptor. Soon afterward, *daf-4* was cloned and shown to encode a protein related to the activin type II receptor (Estevez et al. 1993). It was hypothesized that *daf-1* and *daf-4* act together in the same signaling pathway rather than in two sequential pathways, each requiring a separate TGF- β family receptor.

In addition to causing a dauer phenotype, loss of *daf-4* has the unusual property of also causing small body size (Sma) and male tail abnormalities (Mab). The combination of these phenotypes was unique among the dauer mutants, which suggested that *daf-4* might function in two converging pathways. Further studies confirmed this, showing that DAF-4 is a common type II receptor for the dauer and Sma/Mab pathways. Studies of the Sma/Mab pathway led to the discovery of the *sma-2*, *sma-3*, and *sma-4* genes (Fig. 1) (Savage et al. 1996). Along with the *Drosophila Mad* gene (Sekelsky et al. 1995), the *sma* genes are the founding members of the Smads (Derynck et al. 1996). Given the presence of TGF- β family pathways in *C. elegans*, the use of powerful genetic tools and cell biological techniques have contributed to the elucidation of many elements of the core components of the pathway (Savage et al. 1996; Krishna et al. 1999; Suzuki et al. 1999; Liang et al. 2003; Savage-Dunn et al. 2003; Gleason et al. 2014).

SUMMARY OF *C. elegans* TGF- β -LIKE PATHWAYS

Although there are five TGF- β -related ligands in *C. elegans* (*dbl-1*, *daf-7*, *unc-129*, *tig-2*, and *tig-3*) (Fig. 2), only two complete TGF- β family signaling pathways have been described. Each of these two pathways functions through two serine–threonine kinase receptors and their pathway-specific Smads, as is the case for other

TGF- β family pathways. As introduced above, one of these pathways in *C. elegans* is the dauer pathway, which controls entry and exit from the dauer state, and is induced as a response to harsh environmental conditions; DAF-7 is its ligand. DAF-7 signals through the type I receptor DAF-1 and the type II receptor DAF-4. The Smads for the dauer pathway are recognizable by sequence, but have undergone some structural and functional divergence. DAF-8 and DAF-14 are partially redundant R-Smads, and DAF-14 lacks the MH1 domain. Furthermore, the co-Smad of the pathway, DAF-3, functions antagonistically to the other components. Functions of the dauer pathway in dauer development, aging, and fat metabolism have been characterized and are discussed more fully below. The second TGF- β family pathway, the Sma/Mab pathway, affects body size, male tail development, and other functions (Fig. 3) and signals through the DBL-1 ligand. Each of these pathways uses unique type I receptors and Smads, but both pathways use DAF-4, a type II receptor, rather than having a unique type II receptor for each pathway. The type I receptor for the Sma/Mab pathway is SMA-6. Differences in receptor trafficking between the SMA-6 type I receptor and the DAF-4 type II receptor have been identified in *C. elegans* and are discussed below. The Sma/Mab pathway uses three nonredundant Smad components: SMA-2 and SMA-3 as R-Smads, and SMA-4 as co-Smad. In contrast to many other systems, the co-Smads in *C. elegans* act specifically in one or the other pathway. In this review, we discuss the mechanisms by which the Sma/Mab pathway regulates body size, male tail patterning, innate immunity, mesoderm differentiation, and reproductive aging. Finally, a seventh Smad gene, *tag-68*, was identified by genome sequencing. This gene shows sequence similarity with I-Smads, but its function has not yet been determined, so it has not been assigned to a particular pathway.

DAUER DEVELOPMENT IS REGULATED BY A TGF- β FAMILY PATHWAY

Under harsh environmental conditions, such as high population density, limited food, or high

Figure 1. Generic transforming growth factor β (TGF- β) family signaling pathway through Smads compared with the *Caenorhabditis elegans* dauer pathway and the *C. elegans* Sma/Mab pathway. (A) The classic TGF- β signaling pathway. (B) The dauer pathway. Note that DAF-3, the co-Smad, negatively regulates the pathway and may reside in the nucleus rather than being transported with the R-Smads. DAF-5 is the SnoN/c-Ski ortholog and has only been shown to function in the dauer pathway and not in the Sma/Mab pathway. The inhibitory Smad (I-Smad) TAG-68 is noted with a question mark, as its role in the dauer pathway is not clearly established. (C) The Sma/Mab pathway. The two R-Smads, SMA-2 and SMA-3, associate with SMA-4, the co-Smad, and translocate to the nucleus. One characterized transcription factor, Schnurri, aids in transcription of downstream target genes. The I-Smad TAG-68 is noted with a question mark for the Sma/Mab pathway as well, as its role in this pathway is not clearly established.

TGF- β Family in *C. elegans*

Figure 2. Phylogenetic relationships between *Caenorhabditis elegans* and human transforming growth factor β (TGF- β) family ligands. DBL-1 and TIG-2 cluster in the bone morphogenetic protein (BMP) clade, and DAF-7 and TIG-3 in the TGF- β /activin clade, whereas UNC-129 is more divergent. Sequence alignments and trees were generated using Clustal Omega.

temperature, *C. elegans* can enter the developmentally arrested dauer stage instead of continuously developing through the third larval stage (Golden and Riddle 1984; Hu 2007). Dauer larvae are specialized for long-term survival without feeding. When environmental conditions improve, they resume development into the fourth larval stage and finally adulthood. Large-scale genetic screens for dauer-defective and dauer-constitutive mutants were conducted to identify factors necessary to regulate dauer entry and exit (Swanson and Riddle 1981; Golden and Riddle 1984; Malone and Thomas 1994). These screens identified three key signaling pathways, the TGF- β , insulin, and guanylyl cyclase pathways (Hu 2007).

The genetics of the TGF- β dauer pathway was elucidated early in the 1980s, which gave the dauer pathway a molecular head start in the identification of gene products. From this early analysis, *daf-1* was cloned and shown to be a novel serine–threonine kinase transmembrane receptor (Georgi et al. 1990). It was the first TGF- β family receptor cloned, but given that it was the first one, there were no comparisons or data to implicate it in a TGF- β family pathway; it was viewed as an orphan receptor. Once an activin receptor was cloned (Mathews and Vale 1991), new members in various phyla were identified using comparative genomics and polymerase chain reaction (PCR). DAF-4,

the shared type II receptor for the dauer and Sma/Mab pathways, binds human BMP-2 and BMP-4 (Fig. 4) (Estevez et al. 1993). Subsequent cloning of another gene in the pathway revealed the identity of the ligand to be DAF-7, a TGF- β family member (Ren et al. 1996; Schackwitz et al. 1996). DAF-7 is expressed in favorable environmental conditions and blocks dauer formation.

The dauer pathway signals through two R-Smads, DAF-8 and DAF-14, and a co-Smad, DAF-3 (Fig. 5). DAF-3 is considered the co-Smad of the dauer pathway because of sequence similarity with other co-Smads (Fig. 5) (Patterson et al. 1997). However, all of these Smads have distinct sequence properties, suggesting that they have undergone a more rapid evolution than other members of the family. In particular, DAF-8 is highly diverged (Park et al. 2010), and DAF-14 is missing the MH1 domain (Inoue and Thomas 2000). Due to their highly divergent sequences, DAF-8 and DAF-14 do not cluster as expected with their putative orthologs in a phylogenetic tree (Fig. 5). Closer examination of key residues, such as those in the L3 loop of the Smads (Fig. 6A,B), however, suggests that these components are more related to Smad2 and Smad3 (TGF- β /activin Smads) than to Smad1 and Smad5 (BMP Smads) (Fig. 6A). Because the TGF- β subfamily is a deuterostome invention (Huminiacki et al. 2009; Robertson

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Figure 3. Changes in transforming growth factor β (TGF- β) family signaling in *Caenorhabditis elegans* alter the body size. Wild-type *C. elegans* adults are shown in the middle panel. A decrease in Sma/Mab signaling results in animals that are $\sim 70\%$ as long as wild-type (*top panel*). An increase in Sma/Mab signaling produces animals that are $\sim 20\%$ longer than wild-type (*bottom panel*).

et al. 2015), *C. elegans* does not possess the TGF- β ligand per se, but does have the TGF- β /activin-related signaling components found in all animals (Huminiecki et al. 2009). Loss-of-function *daf-3* mutants are dauer-defective rather than dauer-constitutive, and therefore show a mutant phenotype that is opposite to those resulting from loss of other components of the pathway. This suggests that DAF-3 is negatively regulated by the R-Smads. This antagonistic function is in contrast to the co-Smads of other pathways, which function in the same reg-

ulatory direction as their corresponding receptors and R-Smads. For example, mutations in the co-Smads of the Sma/Mab pathway or the co-Smad in *Drosophila* have the same mutant phenotype as their corresponding R-Smad mutations.

Downstream from the Smads in the dauer pathway, *daf-5* encodes a SnoN/c-Ski homolog (da Graca et al. 2004; Tewari et al. 2004). SnoN and c-Ski were identified as proto-oncogenes that inhibit signaling of Smads in vertebrates (Luo 2004). Mutations in *daf-5* match the phe-

Figure 4. Phylogenetic relationships between *Caenorhabditis elegans* and human transforming growth factor β (TGF- β) family receptors. DAF-4 is a type II receptor; SMA-6 and DAF-1 are type I receptors. Sequence alignments and trees were generated using Clustal Omega.

notypes of *daf-3* mutants, which are dauer-defective. *daf-5* is epistatic to mutations in ligands, receptors, and R-Smads of the dauer pathway (Thomas et al. 1993), thus placing DAF-5 as a possible partner for DAF-3, the co-Smad.

As introduced above, dauer formation in *C. elegans* is regulated by two pathways in addition to the DAF-7 TGF- β family pathway: the guanylyl cyclase and insulin pathways (Hu 2007). These pathways act in parallel, so that double mutants that knockout two pathways at once show more severe defects in dauer formation than single mutants (Thomas et al. 1993). DAF-11 encodes a transmembrane guanylyl cyclase that inhibits dauer arrest and is expressed in chemosensory neurons (Birnby et al. 2000). The insulin/IGF-1 signaling (IIS) pathway is defined by the single insulin receptor in *C. elegans*, DAF-2, which inhibits dauer arrest (Kimura et al. 1997). DAF-2 signals through a canonical kinase cascade including AGE-1 (the ortholog of PI3K; hereafter orthologs will be noted by a slash as in AGE-1/PI3K), PDK-1, AKT-1, and AKT-2 (Hu 2007). The dauer-constitutive phenotypes of mutants defective in these IIS components are suppressed by mutations in genes encoding DAF-18/PTEN and DAF-16/FoxO. Both the IIS and the TGF- β

family signaling components are widely expressed and act nonautonomously to regulate dauer (Apfeld and Kenyon 1998; Inoue and Thomas 2000; Wolkow et al. 2002). Each of the three pathways can be defined by genetic interactions with specific antagonistic downstream components. That is, mutations in genes encoding DAF-3/Smad and DAF-5/SnoN specifically suppress phenotypes of the TGF- β -related pathway, whereas mutations in genes encoding DAF-16/FoxO and DAF-18/PTEN specifically suppress phenotypes of the IIS pathway. In addition, the dauer-constitutive phenotypes of most components of the guanylyl cyclase, TGF- β -related and IIS pathways can be suppressed by dauer-defective mutations in *daf-12*, which encodes a nuclear hormone receptor (Thomas et al. 1993; Antebi et al. 1998). Thus, signaling of a nuclear hormone acts downstream from the other three signaling pathways to integrate and coordinate their inputs into the dauer decision.

Figure 5. Phylogenetic relationships between *Caenorhabditis elegans* and human Smads. The DBL-1 pathway Smads, SMA-2, SMA-3, and SMA-4, are closely related to bone morphogenetic protein (BMP) pathway Smads, whereas the dauer pathway Smads, DAF-3, DAF-8, and DAF-14, are more divergent; TAG-68 is a potential I-Smad of unknown function. Sequence alignments and trees were generated using Clustal Omega.

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Figure 6. L3 sequences of *Caenorhabditis elegans* Smads. (A) Sequence alignment of Smad L3 loops. Amino acid residues in the L3 loop that distinguish bone morphogenetic protein (BMP) R-Smads, transforming growth factor β (TGF- β)/activin R-Smads, and co-Smads are indicated with an asterisk. Despite sequence divergence, DAF-8 and DAF-14 retain molecular determinants of TGF- β /activin R-Smads. (B) Smad structure indicating the MH1 domain, the linker sequence, and the MH2 domain, and the positions of the nuclear localization sequence (NLS), the L3 loop, and the carboxy-terminal phosphorylation motif, SSXS on R-Smads.

THE Sma/Mab PATHWAY FUNCTIONS IN BODY SIZE REGULATION AND INNATE IMMUNITY

The observation that *daf-4* mutants have phenotypes that are not shared with the dauer pathway led to the discovery of a second TGF- β family pathway in *C. elegans*, the Sma/Mab pathway. In this pathway, the molecular underpinnings of the Smads are preserved in *C. elegans*, with a few novel aspects. The small body size phenotype (Sma) and male tail abnormal phenotype (Mab) of *daf-4* led to genetic sleuthing that identified the *C. elegans sma-2*, *sma-3*, and *sma-4* genes (Savage et al. 1996) as genes encoding components of the pathway. Along with the *Drosophila Mad* gene (Sekelsky et al. 1995), these four are the founding members of the Smad family (Derynck et al. 1996). Furthermore, the *C. elegans* studies showed for the first time that multiple Smads function in a single pathway. Two hypotheses were proposed to explain these observations: a heterotrimer model and a sequential action model. Subsequent biochemical analyses of Smad function showed that they function as a heterotrimer. In the

Sma/Mab pathway, SMA-2 and SMA-3 are R-Smads, whereas SMA-4 is a co-Smad. SMA-2 and SMA-3 do not usually substitute for each other, but in some situations, phenotypic differences can be observed, particularly in the innate immunity pathway. Phylogenetic analysis of Sma/Mab pathway components suggests that this pathway is most related to the human BMP pathways.

Body Size

Using the prominent small body size phenotype as an assay, a genetic screen was conducted for candidate mutants in this TGF- β family pathway (Fig. 3) (Savage-Dunn et al. 2003). This screen identified additional alleles of known TGF- β family signaling components, as well as alleles of previously missing components (including the gene encoding the ligand DBL-1). Furthermore, it identified novel components and regulators of this pathway as well as a smaller number of body size regulators in parallel pathways. Additional pathway regulators identified include SMA-9/Schnurri, SMA-10/

LRIG, and ADT-2/ADAMTS, which will be discussed below. Overall, the mutants generated in this screen established the DBL-1 pathway as the principal regulator of body size in *C. elegans*. Mutants in Sma/Mab pathway components have the same number of nuclei as wild-type, indicating that some cells must be reduced in size rather than in number (Nagamatsu and Ohshima 2004). The cell size reduction is not universal, however, because the neuronal soma size is not altered (Goldsmith et al. 2010).

The ligands and receptors of the Sma/Mab pathway are predominantly expressed in three tissues: the pharynx, intestine, and epidermis (Krishna et al. 1999; Yoshida et al. 2001; Wang et al. 2002). To test the requirement for these components in different tissues, defined tissue-specific promoters were used to drive expression of the components in single tissues in genetic mutants for the corresponding component. These experiments showed that epidermal, but not pharyngeal or intestinal, expression is critical for the regulation of body size (Inoue and Thomas 2000; Yoshida et al. 2001; Maduzia et al. 2002; Wang et al. 2002). The largest segment of the epidermis consists of a single multinucleate syncytial cell, hyp7, at the periphery of the animal (Chisholm and Hardin 2005). The epidermis produces and secretes the components of the cuticle, including >170 cuticle collagen isoforms (Page and Johnstone 2007). Cuticle collagen gene expression and cuticle organization are disrupted in Sma/Mab pathway mutants (Liang et al. 2007; Roberts et al. 2010; Schultz et al. 2014). It is intuitively reasonable to ascribe a body-size regulatory function to this tissue, given that it surrounds all other tissues of the animal and that the cuticle is known to regulate body size and morphology. Additional cell-non-autonomous effects likely exist, however, because other parts of the anatomy are also altered in size.

The ligand DBL-1 is expressed primarily in the nervous system, including the ventral nerve cord, from which it signals to target tissues (Suzuki et al. 1999). Directed expression of DBL-1 in a variety of normal and ectopic locations revealed that the specific site of expression is not critical for body size regulation (Savage-Dunn

et al. 2011). Rather, the level of expression correlates with body size, consistent with other evidence that DBL-1 is a dose-dependent regulator of growth (Suzuki et al. 1999).

The genetic screen for body size mutants (Savage-Dunn et al. 2003) identified the gene *sma-9*, which was independently also identified in screens for male tail abnormal and mesodermal patterning mutants (Foehr et al. 2006; Liang et al. 2007). SMA-9 is a large zinc-finger transcription factor related to *Drosophila* Schnurri, which functions with the Smads in the Sma/Mab pathway (Liang et al. 2007). Although SMA-9 is required for all identified functions of the Sma/Mab pathway, *sma-9* mutants often have a less severe or more restricted defect, when compared with mutants in genes for core components of the pathway, indicating some differences in how it interacts. In addition, in the mesodermal patterning function, described below, SMA-9 antagonizes the Sma/Mab pathway (Foehr et al. 2006). The *sma-9* gene undergoes extensive alternative splicing to produce alternative protein isoforms with different numbers of zinc finger domains (Liang et al. 2003). Some of these alternative transcripts have been found to have different functions in vivo (Yin et al. 2010).

Two other genes identified in the screen for small mutants are *sma-10* and *adt-2*. SMA-10/LRIG and ADT-2/ADAMTS are extracellular regulators of the Sma/Mab pathway (Gumienny et al. 2010; Fernando et al. 2011). SMA-10 is a transmembrane protein in the leucine-rich repeat and immunoglobulin domain (LRIG) family, and is capable of binding to Sma/Mab pathway receptors in a cell culture assay (Gumienny et al. 2010). ADT-2 is a secreted metalloprotease in the ADAMTS (a disintegrin and metalloprotease with thrombospondin motifs) family (Fernando et al. 2011). It has both DBL-1-dependent and DBL-1-independent effects on body size, and is required for proper organization of the collagen fibrils in the cuticle. In addition to these positive regulators of the Sma/Mab pathway, an important negative regulator is LON-2/glypican (Gumienny et al. 2007). *lon-2* mutants were not identified in the screen for small mutants, be-

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cause they have an opposite (long body size) mutant phenotype. LON-2 is a transmembrane proteoglycan related to Dally that restricts access of DBL-1 to its receptors, reducing signaling activity (Gumienny et al. 2007).

Innate Immunity

All animals have a sophisticated innate immunity system to protect them from pathogens in the environment (Ermolaeva and Schumacher 2014). *C. elegans* is no exception to this rule, and work in the last two decades has shown that many features of this defense mechanism are conserved with higher vertebrates. Genetic screens identified mutations in *C. elegans* that affect the susceptibility to bacterial infections (Mahajan-Miklos et al. 1999). Subsequent research has defined several signaling pathways that regulate innate defenses, sometimes with overlapping target gene regulation (Schulenburg et al. 2008). Among these master-regulating pathways is the Sma/Mab pathway, which regulates gene expression in the intestine where defenses are mounted against infections. The Sma/Mab pathway aids in resisting infections by *Pseudomonas aeruginosa* and *Serratia marcescens* (Malone and Thomas 1994; Tan et al. 1999; Nicholas and Hodgkin 2004) and perhaps many other bacteria. On infection, some of the highest induced targets of the Sma/Mab pathway are lectins and lysozymes, proteins known to be involved in immune responses (Mallo et al. 2002; Sinha et al. 2012). Many of the genes whose regulation changes on bacterial infection overlap with known downstream targets of the Sma/Mab pathway (Roberts et al. 2010; Sinha et al. 2012).

Sma/Mab PATHWAY REGULATES CELL FATE SPECIFICATION

BMPs function to direct cell fate choices in multiple contexts. For example, in *Drosophila* Decapentaplegic (Dpp) patterns dorsal cells in the developing embryo. In addition, the name “bone morphogenetic protein” derives from the ability of these ligands to induce bone formation. Similarly, in *C. elegans*, DBL-1 func-

tions in the specification of particular cell fates in the mesoderm and the ectoderm.

Mesoderm

The mesoderm, the central layer of the three germ layers in development, gives rise to muscle and various types of nonmuscle tissues. In *C. elegans*, mesodermal tissues arise during embryogenesis and postembryonic development. The *C. elegans* postembryonic mesoderm derives from a blastomere, a precursor cell known as M. The M blastomere divides along the dorsoventral axis to produce cells of the sex muscles and of coelomocytes, which are macrophage-like scavenger cells (Sulston and Horvitz 1977). A role for DBL-1 signaling in mesodermal cell fates was identified by a genetic screen for mutants with alterations in this mesodermal lineage (Foehr et al. 2006). In this genetic screen, alleles of *sma-9*, which encodes the *C. elegans* homolog of Schnurri, were identified (Foehr et al. 2006). Mutations in *sma-9* cause a duplication of sex muscles and a reduction of coelomocytes. Somewhat surprisingly, mutants of the core DBL-1 signaling components show no M lineage defects on their own. Instead, in double mutant combinations with *sma-9*, loss of core DBL-1 components suppresses the *sma-9* M lineage phenotype. This suppression of the M lineage defect suggests that SMA-9/Schnurri antagonizes the TGF- β family pathway to allow for normal M lineage specification (Foehr et al. 2006). The suppression of *sma-9* phenotype provided a powerful way to genetically screen for components and regulators of the DBL-1 pathway. This screen has identified all previously known components of the Sma/Mab pathway, as well as several novel regulators (Liu et al. 2015). One positive regulator from this screen was *drag-1*, which encodes a membrane-associated repulsive guidance molecule (RGM) protein (Tian et al. 2010). Cell culture data in vertebrate models showed that RGM proteins bind as coreceptors for BMP-6 (Babitt et al. 2007; Xia et al. 2008; Andriopoulos et al. 2009; Tian et al. 2010), but the *C. elegans* data provided the first evidence that an RGM protein regulates BMP signaling in vivo (Tian et al. 2010). A sec-

ond factor identified in this genetic screen was the neogenin/DCC homolog UNC-40 (Tian et al. 2013). Like core DBL-1 signaling components and DRAG-1, UNC-40 is a positive regulator of body size and a negative regulator of SMA-9 function in the M lineage. Moreover, in the M lineage, UNC-40 acts independently of netrin, its partner in axon guidance, but requires interaction with DRAG-1, showing a novel role for neogenin in BMP signaling.

The genetic screen for suppressors of the *sma-9* mesodermal phenotype also yielded insight into membrane microdomains that may facilitate signaling. In particular, this screen identified *tsp-21*, which encodes a plasma-membrane localized tetraspanin, as a positive regulator of Sma/Mab signaling (Liu et al. 2015). This tetraspanin was shown to act with the redundantly functioning TSP-12 and TSP-14 in the signal-receiving cells. Mechanistically, TSP-12 and TSP-14 physically associate with the type I receptor SMA-6. Tetraspanins are localized to membrane microdomains enriched in glycosphingolipids. Analysis of mutants defective in steps in the biochemical synthesis of glycosphingolipids shows that at least two of the tested genes promote signaling. These analyses support a model in which tetraspanin- and glycosphingolipid-enriched membrane microdomains positively regulate receptor function, either by modulating receptor localization or trafficking (or both) (Liu et al. 2015).

Ectoderm

The initial categorization of Sma/Mab mutants into a common pathway recognized two phenotypes: Small and Male tail abnormal (Sma and Mab) (Savage et al. 1996). *C. elegans* has two sexual forms: the self-fertile hermaphrodite and the male. The Mab phenotype in males includes defects in the specification of a repeated ectodermal structure: the male sensory rays. In both males and hermaphrodites, two lateral rows of epidermal blast cells known as seam cells divide in a stereotypical pattern in each larval stage and contribute to the large surrounding epidermal syncytium (Hedgecock and White 1985). Specifically in males, the three most pos-

terior seam cells on each side undergo a specialized lineage to give rise to nine sensory structures known as rays (Sulston and Horvitz 1977). Sensory rays contain two neurons surrounded by a structural cell, and function in the detection of the hermaphrodite vulva during mating. The DBL-1 signaling pathway is required to specify the identities of rays 5, 7, and 9. Defects in the specification of ray identities lead to incompletely penetrant fusion of neighboring rays (4-5, 6-7, and 8-9) (Savage et al. 1996; Baird and Ellazar 1999; Krishna et al. 1999; Suzuki et al. 1999). In addition, Sma/Mab mutants are defective in neurotransmitter expression that is characteristic of specific sensory neuron fates (Lints and Emmons 1999; Lints et al. 2004; Siehr et al. 2011). Both the Sma/Mab sensory ray defect and the *sma-9* M lineage phenotype can be interpreted as dorsal-to-ventral transformations in these mutant backgrounds, suggesting that dorsoventral patterning is an ancient and conserved function of BMPs.

Another *C. elegans* TGF- β homolog that functions in male tail development is UNC-129. This ligand was first identified for its function in axon guidance, as described below, and then additionally shown to be expressed in sensory ray neurons in rays 1, 3, 5, and 7 (Nash et al. 2000; Ikegami et al. 2004). Although *unc-129* has no effect on sensory ray development in an otherwise wild-type background, it interacts genetically with other genes required to maintain separate identities in adjacent rays. The likely mechanism is that expression of UNC-129, EFN-4/ephrin-4, and PLX-2/plexin in different subsets of sensory rays maintains their independent identities and prevents their fusions (Nash et al. 2000; Ikegami et al. 2004). These data suggest that *unc-129* is required for cell sorting in the male tail.

Sma/Mab RECEPTOR TRAFFICKING

It has become apparent that receptor trafficking is an important mechanism for regulating the signaling strength and timing of many growth factor pathways (Grant and Donaldson 2009; Sorkin and von Zastrow 2009; Scita and Di Fiore 2010). TGF- β family signaling is no exception,

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but details of its receptor trafficking have only recently been elucidated. There is interplay between signaling pathways and trafficking pathways, each being able to regulate the other to increase or decrease signaling output. Once receptors are internalized, they can be recycled back to the plasma membrane for more rounds of signaling, or be directed to the lysosome for degradation and signal termination. What distinguishes TGF- β family receptor pathways from tyrosine kinase receptor pathways or Notch pathways is the unique property of signaling through a complex of two receptor types, which suggests that additional mechanisms may be in play.

Receptors can enter the cell through clathrin-dependent or clathrin-independent internalization pathways. The clathrin-independent pathways use multiple avenues for internalization, such as caveolin-containing structures, cholesterol-rich domains, and the clathrin-independent carrier/GPI-AP-enriched early endosomal compartment (CLIC/GEEC) pathway, many of which are not well characterized (Fig. 7) (Mayor and Pagano 2007; Grant and Donaldson 2009; Sorkin and von Zastrow 2009; Scita and Di Fiore 2010). It is believed that, in some pathways, signaling occurs in association with early endosomes rather than at the plasma membrane, although this is still

Figure 7. Simplified diagram of the clathrin-dependent pathway. The type I receptors, indicated by a red coiled structure, and the type II receptors, shown as a bifurcated green object, enter the cell through clathrin-coated pits and are transported to the early endosome. At this point, decisions are made whether to be recycled or degraded in the lysosome. Ubiquitylation (Ub) of the receptors plays a role in this decision. The endosomal sorting complexes required for transport (ESCRT) is essential for formation of organelles that destroy ubiquitylated proteins. Hepatocyte growth factor-regulated tyrosine kinase substrate (HGRS-1) is part of the ESCRT complex. Recycling can occur via the recycling endosome or by association with the retromer complex, shown as a red object, with two retromer-associated proteins, vacuolar protein sorting-associated protein 35 (Vps-35), sorting nexin 1 (SNX-1), for transport to the *trans*-Golgi and back to the cell surface.

disputed and difficult to experimentally substantiate. Once internalized, receptors are recycled back to the plasma membrane for additional rounds of signaling or are sent to late endosomes and multivesicular bodies for degradation via the lysosome. Recycling can occur rapidly through early recycling endosomes or through the retromer, a specialized protein complex that shuttles the cargo to the *trans*-Golgi and eventually back to the plasma membrane (Mayor and Pagano 2007; Grant and Donaldson 2009; Sorkin and von Zastrow 2009; Scita and Di Fiore 2010).

The intracellular trafficking events for the TGF- β family are not well defined. Several studies in mammalian cells and *C. elegans* indicate that most TGF- β family receptors internalize through a clathrin-dependent pathway (Ehrlich et al. 2001; Yao et al. 2002; Shapira et al. 2012), whereas another model suggests that receptor signaling occurs through a clathrin-dependent pathway, and degradation occurs through a caveolin-mediated pathway (Di Guglielmo et al. 2003; Chen et al. 2007, 2008). Whether signaling is initiated at the plasma membrane after ligand binding or after internalization in early endosomes has not been clearly elucidated. One possible explanation is that signaling is initiated at the plasma membrane but continues as the complex is internalized.

Trafficking of TGF- β family receptors in *C. elegans* was examined to determine the recycling of both the type I and type II receptors (Gleason et al. 2014). Essentially, all core components of the endocytic pathways are highly conserved in sequence and function from invertebrates to humans. In *C. elegans*, as in mammals, internalization through clathrin-coated pits is observed (Gleason et al. 2014). Using mutants defective in the *C. elegans* AP-2 complex, which prevent type I receptor entry in mammals in clathrin-dependent endocytosis (Sorkin and von Zastrow 2009; Scita and Di Fiore 2010), it was found that the *C. elegans* type I receptor is not internalized (Gleason et al. 2014), consistent with mammalian studies.

Are the type I and type II receptors recycled differently? Perhaps the most important finding from the *C. elegans* receptor trafficking studies

is that the type I and type II receptors are regulated by distinct recycling pathways. Once signaling receptors are internalized, they are trafficked to early endosomes where they can be recycled or eventually degraded by the lysosome. Recycling can occur through an early recycling endosome or through the retromer to the *trans*-Golgi network. That the two receptors recycle differently (Gleason et al. 2014) is suggested by effects of mutant receptor-mediated endocytosis-1 (RME-1) (Grant et al. 2001), which is necessary for recycling to the plasma membrane and recycling from the endosome to the Golgi. The type II receptor accumulates in vesicles, whereas the type I receptor levels are significantly reduced, suggesting degradation. Using a series of endocytic mutants, it was shown that the type I receptor is recycled through the retromer, whereas the type II receptor is not. Biochemical support of these studies is shown from physical binding of the cytoplasmic portion of the type I receptors to the core components of the retromer complex. The recycling of the type I receptor through the retromer is functionally conserved in mammals, as shown in a study in which Beclin 1 recruits the retromer to the type I TGF- β receptor (O'Brien et al. 2015). This puts the type I TGF- β family receptor in a small, but growing class of receptors that use the retromer for recycling back to the plasma membrane. The type II receptor is recycled by a recycling compartment independent of the retromer that contains the small GTPase ADP ribosylation factor 6 (ARF-6) (Gleason et al. 2014).

An additional question is whether these changes in receptor trafficking affect the signaling strength of the pathway. Signaling strength was examined by phenotypic analysis of body size, the use of a nuclear Smad reporter, and two downstream target gene reporters. In *rme-1*, *arf-6*, or retromer mutants, signaling was strongly diminished. For *rme-1* and *arf-6* mutants, animals were only slightly larger than a receptor null as a result of functional inactivation of the pathway, indicating that most signaling was absent. In the case of retromer mutants, the body size was reduced to that of a receptor null mutation, suggesting total loss of signaling.

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This shows that the changes in receptor subcellular localization affect signaling output of the pathway (Gleason et al. 2014).

These data provide an explanation for an old observation that the two receptors have different half-lives (Wells et al. 1997). The type I receptor was shown to have a greater half-life than the type II receptor, and part or all of this difference could be the mechanism by which it is recycled. What purpose could the different recycling mechanisms serve? Because both receptors are required for signaling, this allows for termination of signaling by separating the two receptors, while positioning both receptors for repeated rounds of signaling if needed. The discovery of these separate recycling mechanisms of the two receptors provides a novel avenue for therapeutic intervention to attenuate signaling strength of TGF- β .

TGF- β FAMILY LIGANDS REGULATE AGING AND LONGEVITY

C. elegans is a well-established model for the study of longevity due to its short life span, the ability to generate large sample sizes, and the presence of conserved life-span-regulating mechanisms, such as the insulin signaling pathway. Mutations in the single insulin receptor gene in *C. elegans*, *daf-2*, result in a doubling of life span (Kenyon et al. 1993). Similarly, mutations in components of the TGF- β -related dauer pathway lead to significant increases in life span (Shaw et al. 2007). This effect of this TGF- β family pathway on longevity had been overlooked in earlier studies due to an egg-laying defect in these mutants. Delayed egg laying results in artificially shortened life span because embryos hatch inside the mother leading to matricide. When embryos were prevented from developing by the DNA synthesis inhibitor FUdR, the increased longevity was revealed (Shaw et al. 2007).

The life span extension of *daf-7* mutants requires the activity of DAF-16/FoxO, a transcription factor in the insulin pathway. The role of *daf-7* suggests that the TGF- β family pathway regulates longevity through the insulin pathway (Shaw et al. 2007). In support of this

hypothesis, the TGF- β -related dauer pathway has been shown to regulate the expression of the insulin-like ligand genes *ins-7* and *ins-18*. (Liu et al. 2004; Shaw et al. 2007). Interestingly, in *Drosophila*, reduction in activity of the activin ligand Dawdle or of its downstream Smad, dSmad2, also leads to increased life span, although in this case the TGF- β ligand acts downstream from insulin signaling (Bai et al. 2013). A first indication that TGF- β family ligands also play a role in mammalian aging comes from the anti-aging effects of the TGF- β -related GDF-11 on aging mice (Loffredo et al. 2013; Katsimpari et al. 2014; Sinha et al. 2014).

Unlike the dauer pathway, the Sma/Mab pathway has little or no effect on longevity. In contrast, the Sma/Mab pathway plays an important role in the regulation of reproductive span (Luo et al. 2009, 2010). *C. elegans* hermaphrodites normally cease producing progeny ~ 3.5 days after reaching adulthood, approximately halfway through their adult life spans. Mutants of the Sma/Mab pathway, but not of the dauer pathway, increase reproductive span significantly (for example, to >7 days for *dbl-1* mutants) (Luo et al. 2009). Thus, the Sma/Mab pathway normally restricts reproductive span by regulating the end of the reproductive period. Mechanistically, the Sma/Mab pathway modulates multiple indicators of germline and oocyte quality, including chromosomal nondisjunction, graininess and cavities, and viability of fertilized embryos (Luo et al. 2010). The action of the Sma/Mab pathway on reproductive aging is nonautonomous in the epidermis (a somatic tissue), as shown by tissue-specific expression experiments of SMA-3/Smad (Luo et al. 2010). Interestingly, the regulation of life span and reproductive aging is uncoupled from each other, because *daf-16* and *pha-4* (Luo et al. 2009) are not required for reproductive aging, but are required for the regulation of longevity by DAF-2 IIS and dietary restriction, respectively.

REGULATION OF FAT METABOLISM

In addition to their well-characterized roles in development and differentiation, roles in homeostatic functions are emerging for TGF- β

family ligands. In particular, TGF- β family proteins regulate fat metabolism in several animal species, including *C. elegans* (Ashrafi 2007; Ballard et al. 2010; Zamani and Brown 2011; Ghosh and O'Connor 2014). *C. elegans* does not have dedicated adipocytes, but instead stores fat in the intestine and epidermis. When worms prepare to enter dauer, they store fat for use as a source of energy during the nonfeeding dauer stage (Narbonne and Roy 2009). The major dauer-regulating pathways, acting through DAF-7 TGF- β family signaling, and DAF-2 IIS, are required for this regulation of fat stores in dauer development and also play a role in fat accumulation in other stages. In particular, dauer-constitutive, TGF- β family signaling mutants accumulate more intestinal fat than wild-type animals in adulthood (Greer et al. 2008). Furthermore, like the dauer-constitutive phenotype, the high fat phenotype is suppressed by mutations in *daf-3*, encoding the co-Smad of the pathway. This excess fat accumulation occurs despite reduced feeding. It has not yet been determined whether the TGF- β -related dauer pathway regulates fat accumulation by alterations in fat synthesis, fat breakdown, or both. Nevertheless, synthetic lethality of *daf-7* with *fat-6* and *fat-7*, which encode $\Delta 9$ fatty acid desaturases that are homologous to steroyl co-A desaturase (SCD), suggests that the dauer pathway may have overlapping functions with these critical enzymes involved in fat synthesis (Shi et al. 2013). How this TGF- β family pathway interacts with the insulin pathway in the regulation of fat storage is not yet clear. Unlike the regulation of longevity by the TGF- β -related dauer pathway, regulation of fat accumulation does not depend on DAF-16/FoxO (Ogg et al. 1997). A screen for suppressors specific to the high fat phenotype of *daf-7* mutants identified EGL-30, a subunit of a heterotrimeric G protein, as a modulator of this process (Greer et al. 2008).

UNC-129, A TGF- β FAMILY MEMBER WITH AN UNUSUAL SIGNALING PATHWAY

In addition to the two canonical TGF- β signaling pathways (dauer and Sma/Mab), a noncanonical pathway regulated by TGF- β family

member UNC-129 has been described. UNC-129 was identified in a genetic screen that sought genes interacting with the netrin axonal guidance pathway. UNC-6/netrin is an extracellular guidance protein that mediates axonal pathfinding in invertebrates and in vertebrates (Wadsworth 2002). Two transmembrane proteins serve as netrin receptors: UNC-5 and UNC-40/neogenin. To identify new components of the netrin signaling pathway, a genetic screen for mutations that suppress the effects of ectopic UNC-5/netrin receptor expression was conducted. One of the genes identified in this genetic screen was *unc-129*, which encodes a TGF- β family member. Mutations in *unc-129* cause defects in axonal trajectories of migrating motor neurons (Colavita et al. 1998), similar to mutations in *unc-5* itself, in the genes encoding UNC-6/netrin, and UNC-40/neogenin. *unc-129* is transcribed in muscle of the dorsal body wall, from which the protein is secreted to provide dorsoventral axon guidance cues (Colavita et al. 1998).

Although UNC-129 is a member of the TGF- β family, how it signals is somewhat enigmatic, as the known *C. elegans* TGF- β family receptors do not cause an Unc phenotype, nor do they affect axon migration. This raises the possibility that UNC-129 signals through a nonconventional receptor for the TGF- β family. Intriguingly, UNC-129 physically interacts with UNC-5, a netrin receptor, which suggests UNC-129 functions with UNC-5 (Macneil and Wrana 2009). UNC-5 is not related to TGF- β receptors, as it contains a transmembrane domain with two immunoglobulin-like domains and two extracellular type I thrombospondin motifs (Macneil and Wrana 2009). No binding has been seen between UNC-129 and UNC-40/neogenin, although BMP ligands have been shown to bind neogenin in vertebrate systems (Hagihara et al. 2011). Despite the lack of direct binding, UNC-129 is shown to function by enhancing UNC-5/UNC-40 complex signaling at the expense of UNC-5-alone signaling (Macneil and Wrana 2009). An additional role for UNC-40/neogenin is seen in modulation of DBL-1 activity, as described above, again without direct binding to the ligand (Tian et al. 2013).

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TGF- β FAMILY MEMBERS OF UNKNOWN FUNCTION

Mutations in *tig-2* and *tig-3* have been generated as a result of the nematode consortium efforts to generate mutations in all genes in the organism. Their conservation suggests that they are both functional, but mutant phenotypes have not been described in detail. *tig-2* is expressed in two bilateral neurons in the pharynx, and its loss of function weakly enhances a dauer phenotype (Padgett and Patterson 2008; Gumienny and Savage-Dunn 2013). The location of cell bodies of these neurons suggests that they are the M2 motor neurons (Albertson and Thomson 1976). Given that there are only two complete canonical TGF- β family signaling pathways in *C. elegans*, it is likely that *tig-2* and *tig-3* function through these pathways in either a narrow temporal or spatial window.

ALTERNATIVE SIGNALING MECHANISMS

In the Sma/Mab pathway, most of the identified biological functions require all three Smads: R-Smads SMA-2 and SMA-3, and co-Smad SMA-4. A few exceptions to this rule illustrate possible mechanisms for achieving specificity in signaling outcomes. For example, different Smads are required for the response to different pathogens. The response to the bacterial pathogen *P. aeruginosa* strain PA14 depends on all three Smads, but for a response to the nematophagous fungus *Drechmeria coniospora*, only SMA-3 is needed, not SMA-2 or SMA-4 (Zugasti and Ewbank 2009). Thus, a co-Smad-independent signaling pathway is invoked in the *C. elegans* response to *D. coniospora*. Another example involves a recently discovered role for the Sma/Mab pathway in the pharynx (Ramakrishnan et al. 2014). DBL-1 secretion from the pharyngeal motor neuron M4 regulates pharyngeal gland cell morphology. This regulation of gland cell morphology depends on the receptors SMA-6 and DAF-4, but is independent of Smad activity. It is therefore likely that DBL-1 activity in this context is mediated by an alternative signaling pathway that is yet to be identified. Finally, as mentioned above, the TGF- β

ligand UNC-129 functions through a noncanonical pathway, possibly via the UNC-5 netrin receptor (Colavita et al. 1998; Macneil and Wrana 2009).

CONCLUSION

Genetic studies in *C. elegans* have elucidated many conserved components of TGF- β family signaling pathways and offered mechanistic insights into how they function and are regulated. For example, it was recently shown that the two receptors traffic differently from each other, with the type I receptor recycling through the retromer. All known core components of the TGF- β family signaling pathway are conserved in *C. elegans*, and the general principles learned from *C. elegans* are applicable to vertebrate systems. There are two classic signaling pathways, the Sma/Mab and dauer pathways. UNC-129, a BMP-like ligand, does not signal through either of these two pathways, but may signal through a noncanonical pathway that uses UNC-5 as a receptor.

Although the first biological roles for TGF- β family signaling in *C. elegans* were body size and dauer formation, additional roles have been identified for members of the family. These include innate immunity, mesoderm and ectoderm patterning, longevity, and fat metabolism. Future studies will focus on how TGF- β family signaling impinges on these biological functions. Furthermore, the determinants of tissue-specificity and context-dependence remain to be well understood in any system. The strong genetic, molecular biological, and cell biological tools available in *C. elegans* predict that this system will continue to yield insights into these important and difficult questions.

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