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ARTICLE

The Ortholog of the Human Proto-oncogene ROS1 is Required for Epithelial Development in *C. elegans*

Martin R. Jones,[1]* Ann M. Rose,[1] and David L. Baillie[2]

[1]Department of Medical Genetics, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z4

[2]Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, British Columbia, Canada V5A 1S6

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Summary: The orphan receptor ROS1 is a human proto-oncogene, mutations of which are found in an increasing number of cancers. Little is known about the role of ROS1, however in vertebrates it has been implicated in promoting differentiation programs in specialized epithelial tissues. In this study we show that the *C. elegans* ortholog of ROS1, the receptor tyrosine kinase ROL-3, has an essential role in orchestrating the morphogenesis and development of specialized epidermal tissues, highlighting a potentially conserved function in coordinating crosstalk between developing epithelial cells. We also provide evidence of a direct relationship between ROL-3, the mucin SRAP-1, and BCC-1, the homolog of mRNA regulating protein *Bicaudal-C*. This study answers a longstanding question as to the developmental function of ROL-3, identifies three new genes that are expressed and function in the developing epithelium of *C. elegans*, and introduces the nematode as a potentially powerful model system for investigating the increasingly important, yet poorly understood, human oncogene ROS1. genesis 51:545–561, © 2013 The Authors. Genesis Published by Wiley Periodicals, Inc.

Key words: ROS1 oncogene; *Caenorhabditis elegans*; ROL-3; cuticle; epithelial; seam cells

INTRODUCTION

The advent of high-throughput genomic techniques for identifying mutations on a global scale is revealing the importance of specific proteins that are associated with human diseases, such as cancer. One such protein is the receptor tyrosine kinase (RTK) and proto-oncogene ROS1, the mis-expression of which has been observed in a significant number of human glioblastomas (Birchmeier et al., 1987; Sharma et al., 1989; Birchmeier et al., 1990; Charest et al., 2003). Recently, the growing importance of ROS1 has been highlighted by the discovery of transforming mutations in other cancers types, most significantly in lung (Bergethon et al., 2012; Davies et al., 2012; Janne et al., 2012; Rimkunas et al., 2012; Suehara et al., 2012), and to a lesser degree, breast (Eom et al., 2008), colon and kidney (Ruhe et al., 2007). The discovery of small molecule compounds that can inhibit ROS1, such as Crizotinib, are proving effective for treating specific tumor types (Davies et al., 2012; Komiya et al., 2012), and has led to the proposal that the ROS1 receptor tyrosine kinase is a powerful target for engineered anticancer drugs (Forde and Rudin, 2012; Ou et al., 2012). The identification of ROS1 mutations that are associated with familial heart disease (Shiffman et al., 2005) and hypertension (Yamada et al., 2008) has also implicated this protein in the development of other human diseases beyond cancer.

Although the importance of ROS1 is becoming clear, its developmental role is still poorly understood. ROS1

Additional Supporting Information may be found in the online version of this article.

*Correspondence to: Martin Jones, Department of Medical Genetics, Room 419 NCE Building, 2125 East Mall, University of British Columbia, Vancouver, British Columbia, V6T 1Z4 Canada.
E-mail: jonesmr@mail.ubc.ca

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is conserved in a number of organisms and is one of two remaining orphan receptors, having no known ligand (Acquaviva et al., 2009). Studies in avian and rodent model systems have uncovered a complex spatio-temporal pattern of expression for ROS1 in a range of developing organs including the lung, kidney, liver, intestine, and reproductive tissues (Sonnenberg et al., 1991; Tessarollo et al., 1992; Chen et al., 1994; Sonnenberg-Riethmacher et al., 1996). Expression is highly specific to the epithelial tissues of these organs, and coincides with important morphogenic events (Kanwar et al., 1995). ROS1 −/− transgenic mice are viable, however males are infertile due to improper development of the epididymis (Sonnenberg-Riethmacher et al., 1996), a specialized epithelial structure that regulates sperm development and maturation (for review see Cooper, 2007). In humans, expression of ROS1 is reported in the tissues of a number of organs, including the lungs and the epididymis (Legare and Sullivan, 2004; Acquaviva et al., 2009), revealing potential functional conservation of ROS1 in higher organisms.

The power of model organisms, such as the nematode *C. elegans*, to explore the underlying biology of human disease genes is well established. The epithelium of *C. elegans* is a valuable model system for exploring the function of conserved genes involved in epithelial development (Kagoshima and Shigesada, 2007; Joshi et al., 2010). The outer epithelial system of *C. elegans* is composed of two general cell types: major hypodermal cells and the seam cells, a specialized type of hypodermal cell (reviewed by Altun and Hall, 2009a, b). The largest hypodermal cell (hyp7) is a single multinuclear syncytium that covers most of the main body of the animal. Several smaller hypodermal cells form the head and the tail. The major hypodermis is responsible for establishing the body structure of the animal, and for secretion of cuticle components (Johnstone and Barry, 1996; Greenwald, 1997; Michaux et al., 2001). The seam cells, a specialized group of stem cell-like epithelial cells, also secrete cuticle components and play an additional critical role in the timing of postembryonic development and co-ordination of the molt cycle (Ruaud and Bessereau, 2006; Monsalve et al., 2011; Singh et al., 2011). Seam cells progress through a highly ordered developmental program of cell division, migration, elongation and fusion that leads to the generation of a multinucleate structure running laterally along either side of the animal (Sulston and Horvitz, 1977; Podbilewicz and White, 1994). At the L1 stage, each seam is composed of 10 cells, three anterior head cells (H0–H2), six cells of the main body (V1–V6) and a single tail in the posterior (T). During the L1 stage the seam cells of the V lineage (V1–V6) undergo a symmetric cell division, separating from one another and resulting in a final complement of 16 cells in each seam. The cells of the V lineage act as stem cells, dividing asymmetrically at each of the subsequent larval stages (L2–L4). The anterior daughters of each cell pair fuse with the major hypodermis after each cell division, taking on a hypodermal cell fate. During this process the posterior seam cells elongate in an anterio-posterior direction to retain contact with their neighbors. Upon completion of the hypodermal fusion/seam cell elongation cycle the new cuticle is synthesized and the animal initiates apolysis, the process of releasing the existing cuticle. This is followed by ecdysis, the process of shedding the existing cuticle. Upon completion of ecdysis, seam cell development is re-established. At the last larval stage the seam cells fuse with one another to form a single multinucleate syncytium. This complex developmental process is under precise control of a number of developmental pathways (Ambros, 1989; Ambros, 2001; Abbott et al., 2005; Nimmo et al., 2005; Xia et al., 2007; Joshi et al., 2010; Monsalve et al., 2011; Wildwater et al. 2011). The developmental program of the major hypodermis and seam therefore represents a system in which to study conserved genes that function in the development of epithelial tissues in human diseases, such as ROS1.

The *C. elegans* homolog of ROS1 encoded by the ORF *C16D9.2* was previously identified as ROL-3 (Simmer *et al*., 2003). The developmental function that ROL-3 provides is not known, however mutations in *rol-3* lead to defects in gross morphology that manifest as a roller (Rol) phenotype (Brenner, 1974). Furthermore, in contrast to other Rol loci, ROL-3 also has an essential function in development, as severe alleles result in an early developmental arrest (Johnsen and Baillie, 1991). ROL-3 is of particular interest with respect to ROS1 because the Rol phenotype is known to be associated with defects in the substructure of the cuticle (Peixoto et al., 1998, 2000), structures that are intimately associated with outer epithelial tissues (Page and Johnstone, 2007). Expanding our understanding of how ROL-3 acts to control gross morphology, and confers a phenotype associated with mutation of cuticle collagens, will give insight into important processes in *C. elegans* development, and identify potentially conserved functions with ROS1 in higher organisms.

In this study, we describe the molecular and genetic characterization of ROL-3. We show that ROL-3 is dynamically expressed exclusively in the major outer epithelial tissues of the animal, and is closely associated with the developing seam cells. We find that animals carrying mutations in *rol-3* synthesize a disorganized cuticle, and are defective in molting. Furthermore, we characterize a novel requirement for ROL-3 in the development of the seam syncytium, a tissue associated with the process of molting, and demonstrate that ROL-3 is necessary for the maintenance of the seam cell identity. Finally, we provide evidence of a direct relationship between ROL-3, the predicted mucin SRAP-1, and the Bicaudal-C homolog, BCC-1.

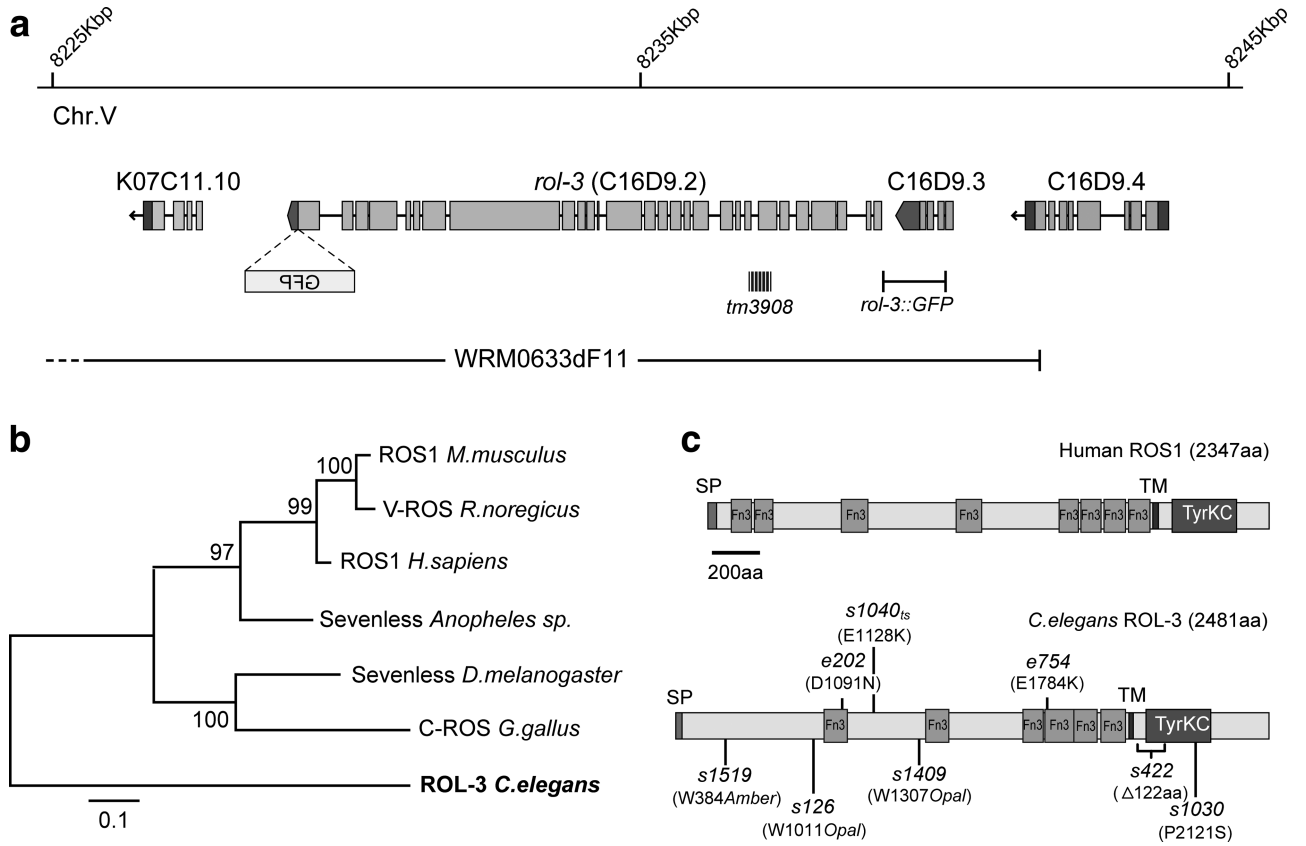

FIG. 1. ROL-3 has a conserved kinase domain and is structurally orthologous to the human proto-oncogene ROS1. **a**. Schematic showing the genomic region of *C16D9.2*. The position of a recombineered C-terminal GFP insertion into the fosmid WRM0633dF11, the genomic region used to drive ROL-3::GFP expression, and the deletion allele (*tm3809*) are show below the gene model. **b**. A closest neighbour tree of *rol-3* derived from BlastP alignment with the putative kinase domain sequence. **c**. Protein schematics of *C. elegans* ROL-3 and Human ROS1. The positions of identified *rol-3* alleles are shown. The alleles *s1040ts* and *e754* have previously been reported (Simmer, Moorman et al. 2003). SP = Signal Peptide, Fn3 = Fibronectin type III repeat, TM = Transmembrane domain, TyrKC = Tyrosine Kinase Domain.

RESULTS

ROL-3 is Structurally Related to the Human Proto-oncogene ROS1

The *rol-3* locus of *C. elegans* encodes a type I integral membrane protein related to the sevenless subfamily of tyrosine kinase insulin receptor genes (Fig. 1b). The structure of the 2481aa protein is typical of an RTK of this class with a single transmembrane domain separating the intracellular and extracellular regions of the protein. The large extracellular portion of the protein contains an N-terminal signal peptide sequence and six fibronectin type III (Fn3) domains. The intracellular region contains a single kinase domain (TyrK; Fig. 1c). BlastP analysis identifies strong similarity between ROL-3 and the human proto-oncogene and orphan receptor ROS1 (29% similarity, 17% identity), particularly within the kinase domain (60% similarity, 35% identity), indicating that ROL-3 is the likely *C. elegans* ortholog of this human proto-oncogene (Acquaviva et al., 2009).

Mutations in *rol-3* Define A Complex Allelic Series

The *rol-3* locus was originally defined by two mutations, *e202* and *e754* (Brenner, 1974), both of which give rise to an adult specific left-hand roller (LRol) phenotype. A further eleven *rol-3* alleles that cause an arrest during larval development were isolated in subsequent screens for essential genes (Johnsen and Baillie, 1991), including a temperature sensitive allele, *s1040ts*. *s1040ts* animals arrest as larvae when grown at 20° but develop into viable adult rollers when cultured at the permissive temperature of 15° (Table 1). The molecular nature of the LRol alleles, *e754* and *s1040ts* has been reported previously (E1822K, E1167K; Fig. 1c) (Simmer et al., 2003). We sequenced the remaining LRol allele, *e202* (D1091N; Fig. 1c). All mutations that give rise to the viable rolling phenotype reside in the region encoding the extracellular portion of the protein (Fig. 1c). We also sequenced five of the remaining alleles, all of which lead to larval arrest. The alleles *s422*, an in-frame deletion of 366bp, and *s1030*, a C-T transition resulting

Table 1
Cuticle Defects in rol-3 Mutant Animals

Allele	Temp.	Terminal stage[a]	Viability (%)	Phenotype
N2 (wildtype)	25°	Viable adult ($n = 100$)	100% ($n = 100$)	WT
rol-3(tm3908)	25°	L2 (100%) ($n = 37$)	0% (n = 37)	Mlt
rol-3(s1040ts)	15°	Viable adult ($n = 148$)	99% ($n = 148$)	LRol
rol-3(s1040ts)	20°	L2 (30%) L3 (70%) ($n = 30$)	0% ($n = 30$)	Mlt
rol-3(s1040ts)	25°	L2 (70%) L3 (30%) ($n = 30$)	0% ($n = 30$)	Mlt
rol-3(s1040ts); sEx2695	20°	Viable adult ($n = 100$)	100% ($n = 100$)	WT

ND = not determined, Mlt – unshed cuticle, CC – cuticle constriction.

[a]Terminal developmental stage assayed by *elt-5::mCherry* or *UNC-47::GFP* expression.

in a Proline to Serine substitution (P2121S; Fig. 1c), are located in the putative kinase domain (Fig. 1c). The remaining three alleles *s126*, *s1409*, and *s1519* are G-A transitions that introduce stop codons (W1011*Opal*, W1492*Amber*, W384*Amber* respectively Fig. 1c). Finally we obtained a UV-TMP generated deletion allele (*tm3908*; Fig. 1a,c; a kind gift from S. Mitani). This mutation is a 324bp deletion that partially deletes exons 7 and 8, resulting in a premature stop at amino acid 432. Observation of *tm3908* homozygotes confirmed that these animals also arrest at an early larval stage (Table 1 and data not shown). All four truncations alleles are predicted to generate proteins lacking both the kinase and transmembrane domains and are therefore likely to be null for ROL-3 function.

ROL-3 is Expressed in a Dynamic Pattern in the Major Hypodermis Throughout Development

To identify the tissues in which *rol-3* is expressed, we generated a transcriptional reporter containing 3kb of the *rol-3* 5′ flanking region fused to the GFP sequence containing a NLS (*rol-3::GFP)*. Animals carrying *rol-3::GFP* as a transgenic array *(sEx1594)* express GFP in all major hypodermal nuclei, with the exception of the seam cells (Fig. 2a–f). Expression is first detected during dorsal intercalation and is particularly intense throughout embryogenesis (Fig. 2a–d). Upon hatching *rol-3::GFP* expression persists in the major hypodermis at a relatively low-level throughout development up until the adult stage, where expression is not detected (Fig. 2e,f and data not shown).

The large size of the *rol-3* ORF made generating a translational reporter by traditional cloning methods technically challenging. To circumvent this we used the recombineering method (Tursun, Cochella et al., 2009) to engineer a fosmid harboring GFP fused in frame at the C-terminus of the gene (Fig. 1a). A transgenic array containing ROL-3::GFP (*sEx2695)* is sufficient for the rescue of all phenotypes associated with mutations in *rol-3*, including the lethality manifested in animals containing the null allele *tm3908* (Table 1 and data not shown). Consistent with the transcriptional GFP reporter ROL-3::GFP is expressed in the major hypodermal cells and excluded from seam cells (Fig. 2g–i). At the point when seam cells are quiescent ROL-3::GFP expression is generally diffuse across the surface of the major hypodermis (Fig. 2j). Prior to and during seam cell division however, ROL-3::GFP expression intensifies and accumulates in foci at the seam cell boundaries (Fig. 2k,l). ROL-3::GFP localization at the seam cell boundary is lost at the anterior seam cells as they fuse with the major hypodermis (Fig. 2k). Particularly intense ROL-3::GFP foci are observed at the leading edges of the posterior seam cells as they elongate toward one another (Fig. 2k). ROL-3 localization at the seam cell boundary persists until seam cell elongation is completed (Fig. 2l). In addition to the major hypodermal cells ROL-3::GFP expression is also detected in the anterior seam cells as they take on the major hypodermal fate at the L1 stage of development (Fig. 2m). These cells migrate to separate the vulval precursor cells (VPCs) which also express ROL-3::GFP at this stage (Fig. 2m). Expression of ROL-3::GFP in the VPC's is restricted to L1 larval stage, being rapidly reduced during the L1–L2 transition and not detected at latter stages of development (Fig. 2m and n and data not shown). The dynamic pattern of ROL-3::GFP localization is consistent with a role in orchestrating crosstalk between the developing seam cells and the hypodermal substrate over which they are developing.

rol-3 is Essential for Proper Cuticle Formation and Molting

In *C. elegans*, Rol phenotypes are associated with mutations that disrupt the biosynthesis, processing and assembly of cuticle collagens (Higgins and Hirsh, 1977; Cox et al., 1980; Levy et al., 1993; Bergmann et al., 1998; Peixoto et al., 1998; Yang and Kramer, 1999; Peixoto et al., 2000). These mutations result in the development of a disordered cuticle structure, arising from improper collagen secretion and assembly. To ascertain if hypomorphic mutations in *rol-3* disrupt cuticle structure we visualized the adult cuticle using the COL-19::GFP reporter, the expression of which can be used as a readout for proper cuticle formation (Thein et al., 2003) (Fig. 3a–d, Supporting Information Fig. S1a,b). In *rol-3(s1040ts)* adults raised at permissive temperature COL-19::GFP is highly disorganized. The annuli,

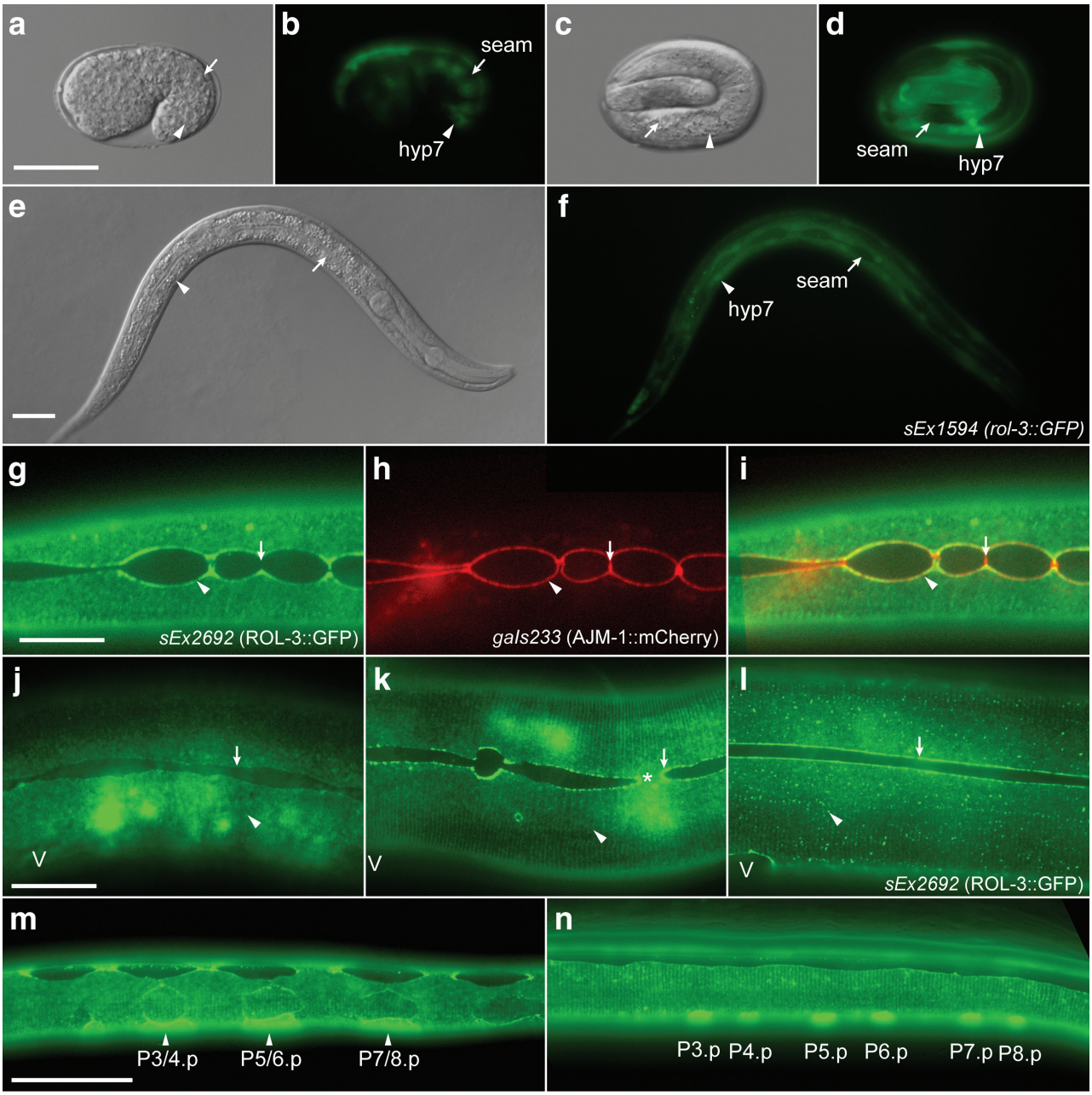

FIG. 2. ROL-3 is expressed in a dynamic pattern in the developing hypodermis. **a–f**, A *rol-3::GFP* transcriptional fusion is expressed in the major hypodermis throughout development. Expression begins at the embryonic stage in most, if not all, major hypodermal cells (arrowheads a–d) and continues throughout post-embryonic development (arrowheads e and f). Expression is not observed in the seam cells (arrows a–f). **g–k**, A dynamic pattern of ROL-3::GFP accumulation is observed throughout post-embryonic development. Broad expression of ROL-3::GFP is seen in the major hypodermis and accumulates in foci adjacent to seam cell boundaries (arrowheads g–i). ROL-3::GFP does not localize with the apical junctions between seam cells (arrows g–i). Prior to seam cell division broad expression of ROL-3::GFP is present in the hypoderm (arrowhead j), but is not present in foci at the seam cell boundary (arrow j). As seam cells divide asymmetrically ROL-3::GFP accumulates at the cell boundaries (arrow **k**). Localization at anterior daughters is lost as they migrate and fuse with the hypodermis (asterisk k). Localization at cell boundaries persists as seam cell elongation is completed (arrow **l**) (V = Vulva). m-n, Strong expression of ROL-3::GFP is observed in VPCs at the L1 (**m**) and early L2 (**n**) stage. Scale bars, 25 μm.

circumferential ridges in the hypoderm that are formed as a consequence of cuticle collagen deposition, form correctly over the dorsal and ventral major hypodermis but are absent from the cuticle in the lateral major hypodermis (Fig. 3c,d). These defects are not specific to the *s1040ts* allele as similar defects are also observed in animals that are mutant for the *e754* allele (Supporting Information Fig. S1a,b). Additionally, *him-8(e1489);*

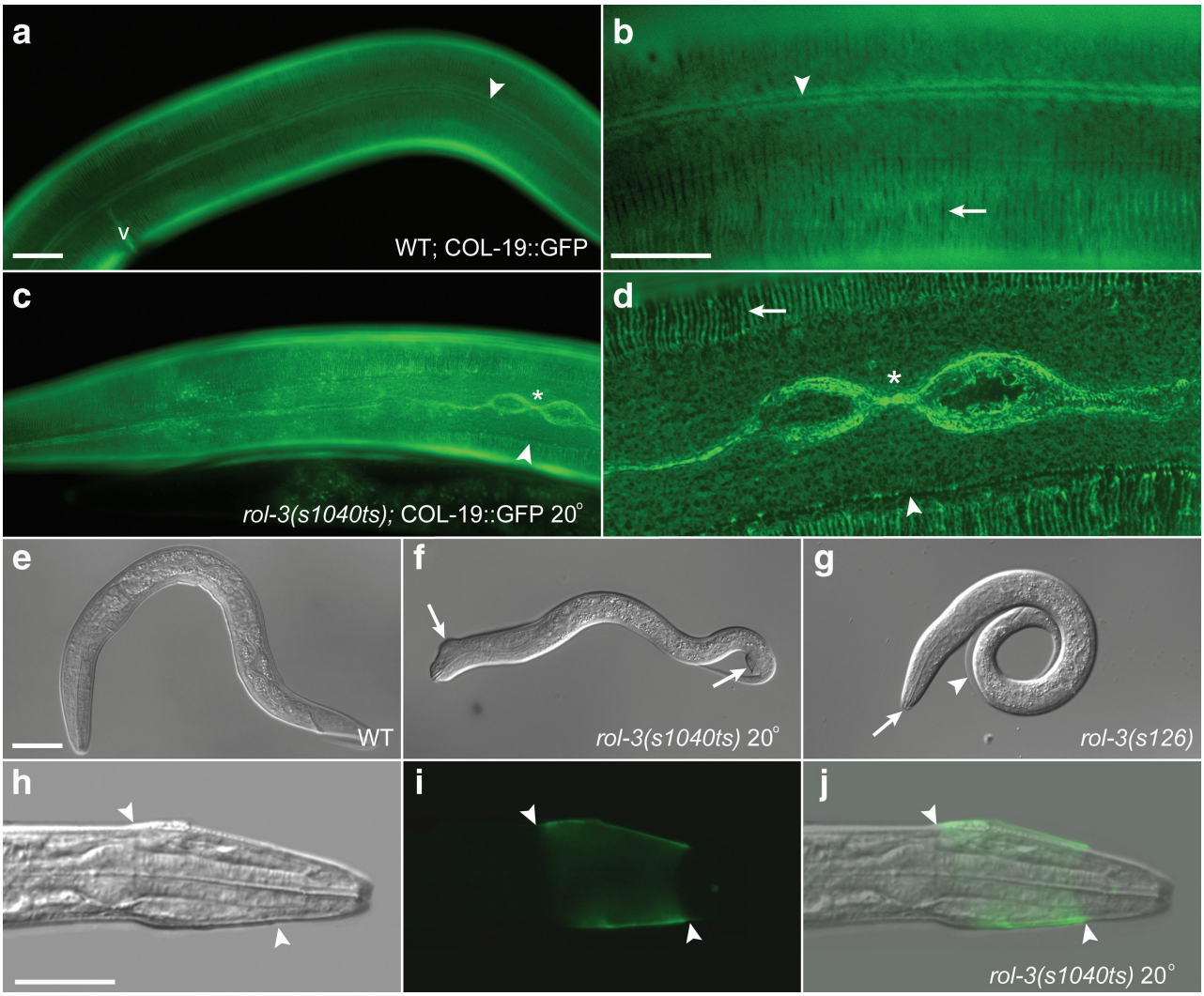

FIG. 3. Mutation of *rol-3* leads to defects in cuticle structure. a-d. COL-19::GFP expression. **a** and **b**. COL-19::GFP expression reveals the ordered structure of the alae (arrowhead) and annuli (arrow) in the cuticle of adult wildtype animals. **c** and **d**. In *rol-3(s1040ts)* adults expressing COL-19::GFP the alae are disrupted (arrowhead) and annuli do not extend across the lateral region of the cuticle (arrow). Bifurcation of the alae is also observed (asterisks). **e–j**. *rol-3* larval cuticle defects. E. L2 stage WT animal. F. Arrested *rol-3(s1040ts)* animal presenting with morphology defects (arrows). G. Arrested *rol-3(s126)* animal fully encased in unshed cuticle (arrowhead and arrow). **h–j**. FITC-WGA staining of an arrested *rol-3(s1040ts)* L1–L2 animal. Unshed cuticles can be seen resulting in a characteristic constriction (arrowhead). Fluorescently labelled WGA binds the exposed and unshed L1 and L2 cuticles beneath (green). Scale bars, 25 μm.

rol-3(e754) animals display gross morphological defects of male specific structures, consistent with improperly formed or accumulated cuticle (Supporting Information Fig. S1c–d). Furthermore, breaks and bifurcations occur in the Alae, cuticular structures that form over the lateral seam cells (Fig. 3c,d and Supporting Information Fig. S1b). Hypomorphic mutations in *rol-3* therefore give rise to cuticle defects consistent with disruption of collagen processing and assembly.

The cuticle defects associated with hypomorphic mutations of *rol-3* could account for the adult specific rolling phenotype. However, the cause of the larval arrest associated with more severe alleles of *rol-3* was unclear. *rol-3(s1040ts)* animals arrest as larvae when grown at restrictive temperature, but are superficially wildtype in appearance. Closer examination of these arrested animals revealed the presence of subtle morphological defects and a distinctive anterior constriction of the cuticle, consistent with the retention of an incompletely shed cuticle (Fig. 3f–j and Table 1). To examine the integrity of the cuticle in these animals we stained arrested *rol-3(s1040ts)* larvae with fluorescein isothiocyanate conjugated wheat germ agglutinin (FITC-WGA). Similar probes are known to preferentially bind glycoproteins exposed by cuticle damage (Politz et al., 1990). FITC-WGA staining of arrested *rol-*

3(s1040ts) larvae confirmed the presence of improperly shed cuticles (Fig. 3h–j). Furthermore, examination of animals homozygous for the null allele *rol-3(s126)* revealed that these animals appear to be fully encased in unshed cuticle (Fig. 3g). Together these observations demonstrate that both the early larval arrest and late-onset LRol phenotypes are associated with defects in cuticle formation and function.

ROL-3 is Required for the Fidelity of Seam Cell Elongation and Morphogenesis

The molting defects associated with null *rol-3* alleles might be due to the inability to release an improperly formed cuticle. Molting defects are also often associated with mutations that lead to the disruption of seam cell morphology (Brooks et al., 2003; Silhankova et al., 2005; Ruaud and Bessereau, 2006; Meli et al., 2010; Monsalve et al., 2011; Singh et al., 2011). Our examination of COL-19::GFP expression in *rol-3(s1040ts)* adult animals revealed the presence of defects in the patterning of the alae, the formation of which are dependent on the structure of the underlying seam syncytium (Page and Johnstone, 2007; and reviewed by Altun and Hall, 2009a, b). This indicated that formation of seam syncytium might be compromised in these animals. To observe the developing seam syncytium we utilized the transgenic array *wIs78*, which expresses GFP in seam cell nuclei and adherin junctions (Li et al., 2005). In *rol-3(s1040ts)* and *rol-3(e754)* larvae raised at permissive temperature seam cell elongation progresses normally or with minor defects in seam cell contact (Fig. 4b and Supporting Information Fig. S1e). Adult animals develop a seam syncytium that can be mildly disorganized, bifurcated and/or discontinuous (Fig. 4d, Supporting Information Fig. S1f and Table 2). The specification of seam cell identity is not significantly affected in *rol-3(s1040)* animals, based on the number of seam cells displaying robust expression of SCM::GFP (Fig. 4d and Table 2). However, *rol-3(s1040ts)* animals arrested at the restrictive temperature of 20° exhibit severe defects in seam formation, with many cells failing to elongate correctly (Fig. 4d/e and Table 2). All animals observed present with these defects, though the severity of the phenotype varies from those that complete cell contact in all but a few cells, to those where cell contact fails completely (Table 2). In animals homozygous for the null allele *rol-3(s126)*, seam cells completely fail to elongate toward one another, retaining a rounded appearance (Fig. 4g/f). Additionally, seam cell number appears to be reduced in both *rol-3(s1040)* and *rol-3(s126)* animals (Fig. 4d, g).

ROL-3 is Required for the Maintenance of Seam Cell Identity in the Developing Seam Syncytium

To further explore the defects we had observed in seam cell morphology we used an *elt-5::mCherry* fluorescent reporter (Liu et al., 2009) to follow the fate of anterior and posterior seam cells. *elt-5* encodes for a transcription factor that is expressed exclusively in seam cells (Koh and Rothman, 2001). Unlike SCM::GFP, *elt-5::mCherry* expression persists in the anterior daughters of the seam cells upon their fusing with the hypodermal syncytium. *rol-3(s1040ts)* and *rol-3(tm3908)* animals expressing *elt-5::mCherry* hatch with the correct number of *elt-5::mCherry* positive cells (Table 2). However, after exit from the L1 stage expression of the reporter becomes erratic and is frequently lost in individual seam cells (Fig. 4h, i and Table 2). Loss of *elt-5::mCherry* expression is more common in *rol-3(tm3908)* null animals, but is not eliminated entirely (Fig. 4i). Seam cells that retain *elt-5::mCherry* expression continue to divide normally (Fig. 4h, i). In *rol-3(tm3908)* animals, loss of *elt-5::mCherry* expression is observed in all P V and T seam cell lineages. However, losses occur more frequently in the V lineage seam cells of the central body (Fig. 4j). Together these observations indicate that ROL-3 is not required for establishing seam cell identity *per se*, but rather is needed for the maintenance of seam cell identity.

The Suppressors of *rol-3*, *bcc-1*, and *srap-1*, are Expressed in Epithelial Tissues

We have previously reported the identification of deletions of *bcc-1*, and duplications of *srap-1* that act to suppress the lethality associated with *rol-3(s1040ts)* animals (Jones et al., 2012). *bcc-1* encodes a homolog of the drosophila RNA binding protein Bicaudal-C (BIC-C), which acts to negatively regulate the function of specific genes by binding to and targeting their mRNAs for degradation (Saffman et al., 1998; Chicoine et al., 2007). *srap-1* encodes a predicted mucin, a family of high molecular weight extracellular matrix (ECM) proteins that form a protective barrier secreted by many cell types, including epithelial cells (Gendler and Spicer, 1995; Chakraborty et al., 2011). It is unclear how the mutations in *bcc-1* and *srap-1* elicit their suppressive effect. As an ortholog of Bic-C, BCC-1 might provide a post-translational regulatory function, and its loss of function would therefore be expected to lead to the overexpression of specific genes through persistence of their mRNA species. Similarly duplications of *srap-1* might result in an overexpression of the protein, also resulting in a gain of function phenotype. It is possible that potential overexpression phenotypes might compensate for the loss of ROL-3 function in hypodermal tissues.

To ascertain if BCC-1 and SRAP-1 might function in the epithelium we first determined their expression patterns. A transcriptional GFP reporter driven by the putative promoter region of *bcc-1* is expressed exclusively in the major hypodermis throughout development (Fig. 5a). This pattern of expression is similar to the expression pattern of *rol-3* and is consistent with a potential

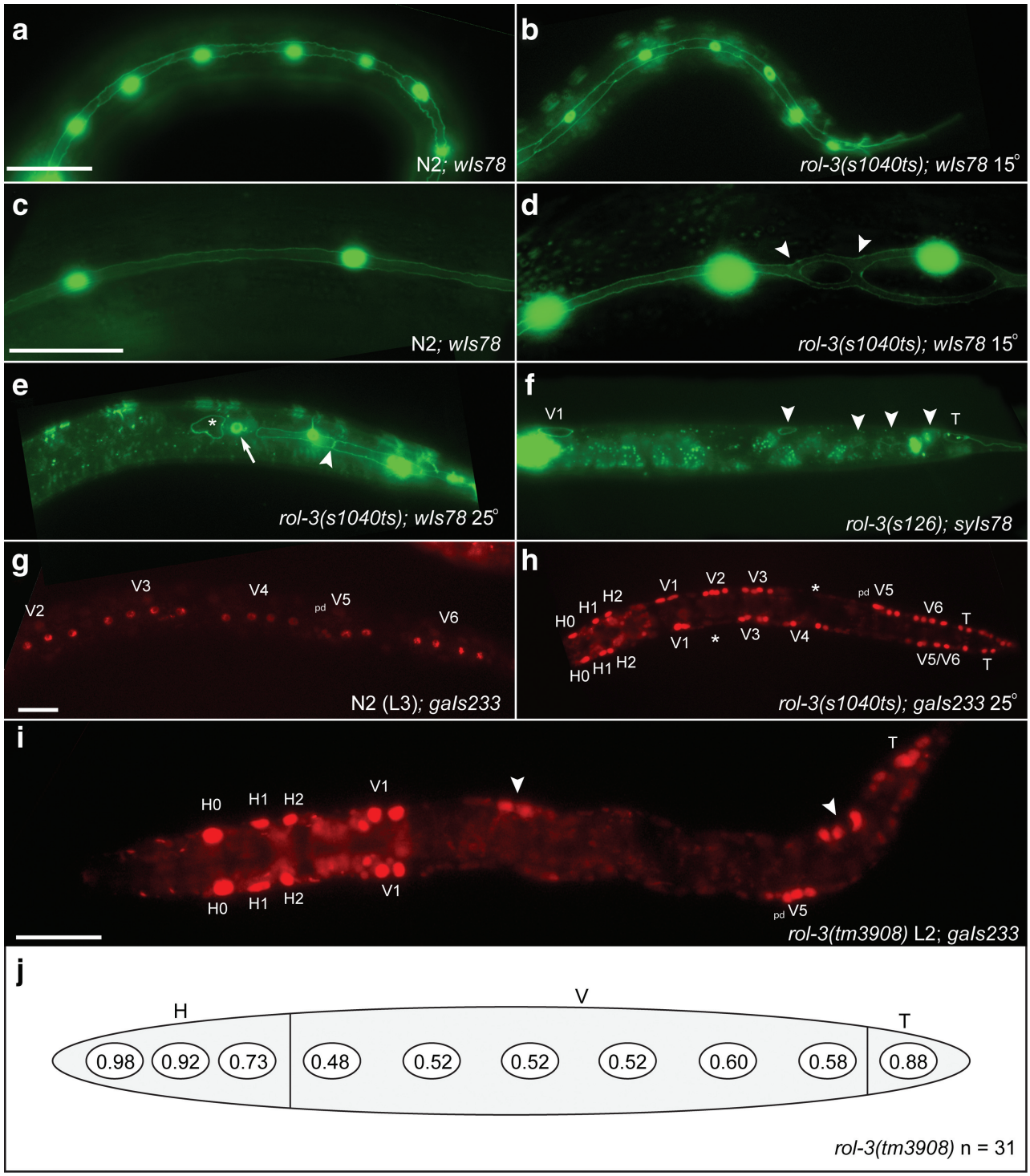

FIG. 4. Development of the seam syncytium is perturbed in animals carrying mutations in *rol-3*. **a**. Wild type L2 stage animal showing the characteristic ladder structure of the seam cells. **b**. *rol-3(s1040ts)* L2 animal raised a permissive temperature displaying a superficially wild type seam structure. **c**. WT adult seam syncytium is narrow and complete (arrow). **d**. Adult *rol-3(s1040ts)* animal displaying a disordered seam that is bifurcated (arrowheads). **e**. Arrested *rol-3(s1040ts)* animal. Two seam cells have elongated correctly (arrowhead) while an adjacent SCM::GFP expressing seam cell has ectopically fused with the hypodermis (arrow). A seam cell that has lost SCM::GFP expression and not elongated can be seen (asterisk). Several seam cells in the anterior of the animal have lost SCM::GFP expression and fused with the hypodermis (left image). **f**. Arrested *rol-3(s126)* animal showing a reduced number of AJM-1::GFP positive seam cells that have not elongated, note: several seam cells are not in the focal plane in this image (arrowheads). **g**. WT L3 animal expressing seam specific *elt-5::mCherry*. **h**. Arrested *rol-3(s1040ts)* animal with an L3 equivalent number of divided *elt-5::mCherry* positive cells. Note that the animal is much smaller than wildtype. Several cells of the V lineage have lost of *elt-5::mCherry* expression (asterisks). i. *rol-3(tm3908)* animal arrested approximately at the L3 stage showing severe loss of *elt-5::mCherry* specification in the V lineage J. Quantification of the loss of seam cell specification in the H, V and T seam cell lineages in arrested *rol-3(tm3908)* animals. Scale bars, 25μm.

Table 2
Seam Cell Defects in rol-3 Mutants

Allele	*T* (°C)	L1 seam #[a]	Terminal seam #[b]	Viability	WT seam cell elongation (%)	Seam cell bifurcation (%)	Seam cell breaks (%)	SCM expression
N2	20	10 (*n* = 40)	15.9 (*n* = 37)	100 (*n* = >100)	100 (*n* = 37)	<1	0	Robust
tm3908	20	10 (*n* = 29)	6.6 (*n* = 31)	0 (*n* = 76)	0 (*n* = 31)	NA	NA	ND
s1040ts	15	10 (*n* = 30)	15.3 (*n* = 65)	99 (*n* = >100)	90 (*n* = 65)	75	10	Robust
s1040ts	20	10 (*n* = 30)	ND	0 (*n* = >100)	0 (*n* = 34)	NA	NA	Variable
s1040ts	25	10 (*n* = 30)	ND	0 (*n* = >100)	0 (*n* = 38)	NA	NA	Variable
bcc-1(tm3921)	20	ND	ND	100 (*n* = >100)	100 (*n* = 78)	0	0	ND
bcc-1(tm3821); rol-3(s1040ts)	20	ND	ND	42 ([d])	0 (*n* = 35)	63.5	13.6	ND
bcc-1(tm3821); rol-3(s1040ts)	25	ND	ND	0 (*n* = >100)	0 (*n* = 59)	0	100	ND
bcc-1(tm3821); rol-3(s1519)	20	ND	ND	0[c]	ND	ND	ND	ND
srap-1(sDp33); rol-3(s1040ts)/+	20	ND	ND	100 (54)	100 (*n* = 24)	0	0	ND
sDp33; rol-3(s1040ts)	20	ND	ND	96 ([d])	0 (*n* = 83)	24.1	1.2	ND
sDp33; rol-3(s1040ts)	25	ND	ND	0 (*n* = >100)	0 (*n* = 41)	0	100	ND
sDp33; rol-3(s1519)	20	ND	ND	0[c]	ND	ND	ND	ND

ND = not determined.

[a]L1 stage seam cell number assayed by *elt-5::mCherry* expression.

[b]Adult seam number assayed by *SCM::GFP* expression.

[c]No viable double mutant animals recovered in genetic crosses (see Methods).

[d]Jones, Rose et al. 2012.

role for BCC-1 in modulating mRNA species in the developing epithelium. A transcriptional GFP reporter driven by the putative *srap-1* promoter is detected in a variety of tissues including the major hypodermis and seam cells (Fig. 5b,c). Notable expression of this reporter is also observed in the tissues of the developing vulva and central nervous system (Fig. 5d,e). To gain further insight into the expression of *srap-1* we used recombineering to generate a fosmid containing GFP fused in-frame at the C-terminus of the *srap-1* ORF. Expression of SRAP-1::GFP is detected at the surface of the newly synthesized cuticle coincident with the molt (Fig. 5f). Significant accumulation of SRAP-1::GFP is also detected at the seam syncytium in the adult stage (Fig. 5g,h). Given that both *bcc-1* and *srap-1* are expressed in epithelial tissues we asked if this expression is dependent on ROL-3 function. *rol-3(s1040)* mutant animals raised at the restrictive temperature of 25° display robust expression of both the *bcc-1::GFP* and SRAP-1::GFP reporters (Supporting Information Fig. S2a–d). Together this expression pattern analysis reveals that both *bcc-1* and *srap-1* are expressed in epithelial tissues associated with ROL-3 function or localization, and that this expression does not appear to be dependent on the expression of *rol-3*.

Suppression of *rol-3*-Associated Lethality by the Mutations *bcc-1(tm2831)* and *srap-1(sDp33)* is Dependent on the Presence of Partial ROL-3 Function

The mutations *bcc-1(tm3821)* and *srap-1(sDp33)* suppress the lethality associated with the temperature sensitive allele *rol-3(s1040ts)* when grown at 20°. We investigated seam cell development in the double mutant animals to determine if this suppression might arise from a restoration of seam cell development. Animals mutant for either *bcc-1(tm2831)* or *srap-1(sDp33)* alone do not display any obvious seam cell defects, indicating that they are not required for the development of this tissue (Fig. 5i,l and Table 2). However, *rol-3(s1040ts)* animals doubly mutant for the suppressor mutations develop a highly disorganised and ectopically formed seam syncytium when grown at 20° (Fig. 5j,m and Table 2). This observation suggested that either the suppressor mutations are promoting seam cell development in the absence of ROL-3 function, or alternatively, they are directly or indirectly promoting the function of a hypomorphic *rol-3* product. To test between these two possibilities we determined whether the suppressor mutations would rescue lethality in animals that are null for *rol-3* function. We find that *bcc-1(tm3921)* and *srap-1(sDp33)* mutations are unable to suppress lethality in animals carrying the null *rol-3(s1519)* or *rol-3(tm3908)* alleles (Table 2). Furthermore, we find that suppression is abolished when animals doubly mutant with the *rol-3(s1040ts)* allele are cultured at a higher temperature of 25°. Seam cell elongation is completely abrogated in these animals, leading to a developmental arrest and severe molting defects (Fig. 5k,n, Table 2). Together these observations indicate that the suppression of lethality is dependent on partial ROL-3 function, and also implies that *rol-3(s1040)* animals cultured at 25° are essentially null for ROL-3 function.

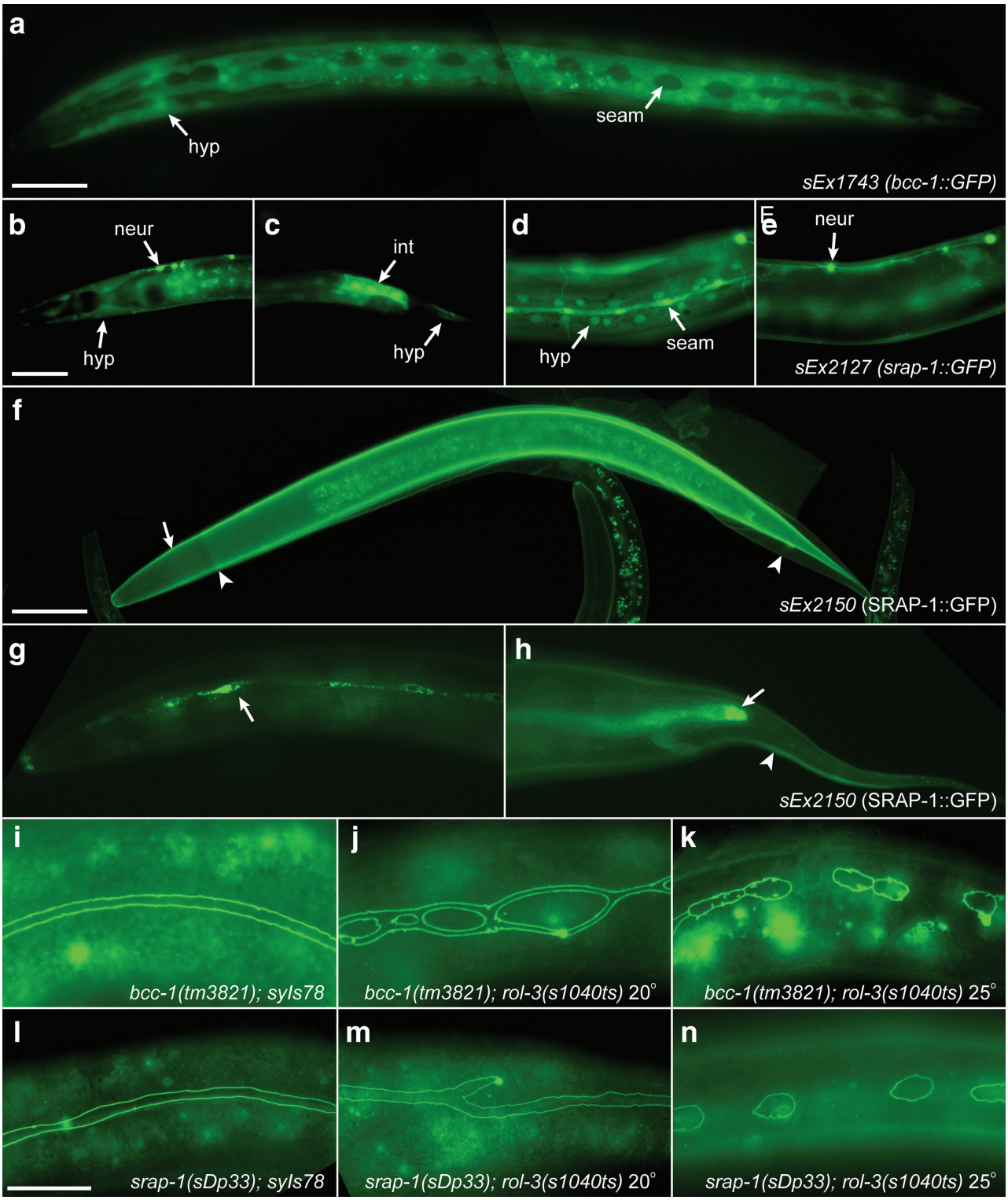

FIG. 5. Suppressors of ROL-3 are expressed in epithelial tissues and modify seam cell defects. **a**. *bcc-1::GFP* is expressed exclusively in the major hypodermis (hyp) being excluded from the seam cells (seam). **b–e**. *srap-1::GFP* is expressed dynamically in many developing tissues throughout development including the larval (b and c) and adult (d and e) hypodermis, seam cells, intestine (d), and neurons. Note that the same animal in different focal planes is shown in panels d and e. **h–j**. SRAP-1::GFP, is secreted at the newly synthesized cuticle (arrow) coincident with the release of the previous cuticle (arrowheads) (**f**) and accumulates at the seam cells in adults (arrows g and h). I–**n**. *bcc-1(tm3821)* and *srap-1(sDp33)* promote seam cell elongation in *rol-3(s1040ts) animals*. *bcc-1(tm3821)* and *srap-1(sDp33)* animals develop a wildtype seam syncytium (i and l). *bcc-1(tm3821); rol-3(s1040ts)* and *srap-1(sdp33)*; *rol-3(s1040ts)* double mutant animals display disorganised and ectopic seam cell development at 20° (j and m). In double mutants at 25° seam cell elongation is severely abrogated (k and n). Scale bars, 25 μm.

DISCUSSION

In this study we have investigated the developmental role of the essential receptor tyrosine kinase ROL-3. We have shown that mutations in *rol-3* lead to improper molting and the development of a compromised adult cuticle, phenotypes that are indicative of the LRol phenotype. We have also established that ROL-3 is expressed in the major hypodermis in a dynamic manner, where it forms foci at the boundaries of the developing seam cells. Furthermore, we have demonstrated that ROL-3 is required for seam cell development and the maintenance of seam cell identity. Finally we provide evidence that the suppressor mutations *bcc-1(tm3821)* and *srap-1(sDp33)* act to modify seam cell development in a manner that is dependent on partial ROL-3 function.

We find that animals carrying hypomorphic mutations in *rol-3* have a compromised cuticle. Viable adult *rol-3(s1040ts)* animals display defects in collagen disposition specifically over the lateral hypodermis. This might account for the observed adult specific rolling phenotype as, with the exception of mutations in *rol-3*, all known mutations that manifest as a LRol phenotype reside in cuticle collagens (Kramer et al., 1988, 1990; Levy et al., 1993; van der Keyl et al., 1994; Novelli et al., 2004). Additionally we have demonstrated that ROL-3 is expressed in the major hypodermis where many cuticle collagens are synthesized (Page and Johnstone, 2007). Together these observations highlight a potential link between ROL-3 function and the biogenesis of cuticle collagens in the major hypodermis. The LRol phenotype has been shown to arise due to specific mutations that affect the processing and trimerization of procollagens (Yang and Kramer, 1999). As a putative signaling molecule ROL-3 could potentially function to directly or indirectly co-ordinate the biogenesis and incorporation of collagens into the cuticle.

In addition to the LRol phenotype, we have demonstrated that null alleles of *rol-3* result in developmental arrest, associated with an inability to molt. Molting is distinctly associated with the integrity of the seam syncytium as disruption of seam cell development is associated with molting defects (Koh and Rothman, 2001; Brooks et al., 2003; Silhankova et al., 2005; Hao et al., 2006; Ruaud and Bessereau, 2006; Fritz and Behm, 2009; Monsalve et al., 2011; Singh et al., 2011). Furthermore, molting requires numerous proteases, steroid hormones, and nuclear hormone receptors (Yochem et al., 1999; Gissendanner and Sluder, 2000; Kostrouchova et al., 2001; Brooks et al., 2003; Kuervers et al., 2003; Davis et al., 2004; Frand et al., 2005; Kim et al., 2005; Hayes et al., 2006; Thein et al., 2009; Stepek et al., 2010; Kim et al., 2011), many of which are produced specifically in the seam cells. We observe defects in seam cell formation in animals carrying mutations in *rol-3*. In these animals the seam cells appear to be competent for developmental processes such as cell division and fusion with the surrounding hypodermal tissue. However, defects in elongation of posterior seam cells are observed as the cells attempt to retain contact with one another. The severity of these defects is directly related with the severity of the *rol-3* mutation; subtle defects in seam cell morphology are observed in animals carrying the hypomorphic mutations *rol-3(e754)* and *rol-3(s1040ts)*, while elongation is completely abrogated in animals carrying the null mutations *rol-3(tm3908)* and *rol-3(s126)*. Together these observations indicate that ROL-3 is required to promote elongation of seam cells as they form connections with one another.

The pattern of expression and localization that we have described indicates that ROL-3 acts in a noncell-autonomous manner to influence the development of the seam syncytium. This might be surprising given that as a signaling molecule ROL-3 is expected to elicit an effect within the major hypodermis where is it expressed. As the seam syncytium is embedded within the major hypodermis its formation likely requires that the developing seam cells navigate the surface of this cell in order to establish and maintain contact with one another. A signaling mechanism mediating cross-talk between the seam cells and the major hypodermis might therefore be required to establish or modify the substrate over which the seam cells can develop. The activation of ROL-3 by a signal from the developing seam cells might represent the first step in a signal cascade leading to the establishment of a permissive substrate for seam cell development. It has been noted that no major signaling molecules that could facilitate cross-talk between the major hypodermis and the seam syncytium have been identified in large-scale screens for genes involved in molting (Frand et al., 2005). It is tempting to speculate that such a role could be provided by ROL-3. This hypothesis is supported by the observation that ROL-3 localizes dynamically at the boundary between the major hypodermis and the developing seam cells. A notable example of another gene in *C. elegans* that is expressed in the major hypodermis but disrupts alae formation, and by extension seam cell development or function, is *cut-6*, which encodes a novel cuticulin important for body morphology (Muriel et al., 2003). However we cannot rule out the possibility that ROL-3 also functions within the seam cells themselves eliciting a direct effect on seam cell morphology and development. Further work is needed to determine the precise mechanism of ROL-3 function.

In addition to seam cell morphological and developmental defects, we also describe the loss of seam specific markers in animals carrying mutations in *rol-3*. *rol-3* mutant animals, including those harboring null alleles, hatch with the correct number of seam cells,

demonstrating that ROL-3 function is not required to establish seam cell identity. However, during subsequent postembryonic development a gradual loss of seam cell identity is observed. The loss of seam cell identity is more severe in animals carrying the null allele *rol-3(tm3908)* but not eliminated entirely. The loss of seam cell specification therefore appears to occur stochastically. A hypothesis for these observations is that reestablishing a physical connection after cell division is a requirement for continued maintenance of seam cell identity. Seam cell identity is dependent upon a number of distinct developmental mechanisms including; miRNA mechanisms of post transcriptional control, Runx, and engrailed-type transcriptional regulation, and both canonical and noncanonical Wnt signaling (reviewed by Joshi et al., 2010). In the absence of ROL-3 mediated seam cell development these cues might be uncoupled, leading to loss of fate and ectopic fusion with the major hypodermis. In support of this hypothesis seam cell ablation studies have shown that formation for the posterior deirid from the V5 seam cell lineage is dependent up contact being made between adjacent seam cells (Austin and Kenyon, 1994). Additional studies to characterize the relationship between cell contact and the maintenance of seam cell identity using mutant alleles of *rol-3* are likely to be informative.

We have shown that the suppressors of *rol-3*-associated lethality, BCC-1 and SRAP-1, exacerbate seam cell defects in a ROL-3 dependent manner. Mutations in *bcc-1* and *srap-1* were identified in a suppressor screen for mutations that promote survival of *rol-3(s1040ts)* animals at the restrictive temperature of 20° (Jones et al., 2012). When cultured at 25° however this suppression is completely abrogated. We also find that suppressor mutations are unable to supress lethality in null animals. Given that animals carrying *rol-3* mutations arrest trapped within unshed cuticles it is possible that the suppressor mutations promote cuticle release, allowing animals with minimal ROL-3 function to progress in development. Consistent with this hypothesis we have shown that SRAP-1 is secreted into the cuticle during molting. Furthermore suppression by *srap-1* is facilitated by duplications of the gene, suggesting a gain of function phenotype. Similarly, as a putative regulator of specific mRNAs, loss of BCC-1 in the major hypodermis might act to increase molting cues or the production of enzymes that promote cuticle release. An alternative model is that suppressors might act directly or indirectly to promote increased function of a disabled *rol-3* product. Further investigation is needed to confirm the suppressive relationship between BCC-1, SRAP-1 and ROL-3.

ROL-3 is the closest homologue in the *C. elegans* genome to the human proto-oncogene ROS1. The endogenous function of ROS1 is not well known, however in the murine model ROS1 is specifically expressed in epithelial tissues where it is required for the development of the epididymis. Expression of ROL-3 in epidermal tissues is consistent with the murine model, demonstrating the potential for a conserved function between these proteins. It has been demonstrated that ROS1 directly binds SH2 domain containing tyrosine phosphatase SHP-1 (Keilhack et al., 2001), and has been shown to activate both SHP-1 and SHP-2, the mitogen-activated protein kinase ERK1/2, insulin receptor substrate 1 (IRS-1), phosphatidylinositol 3-kinase (PI3K), protein kinase B (AKT) and the STAT3 and VAV3 signaling pathways (Zong et al., 1993; Xiong et al., 1996; Zeng et al., 2000; Charest et al., 2006). Orthologs of several of these proteins are present in the *C. elegans* genome, leading to potential future studies to test the conserved nature of these interactions. Furthermore, like ROS1, ROL-3 is an orphan receptor having no known ligand. Given the analysis we have presented here, the seam syncytium is the likely source of the ROL-3 ligand.

Finally ROS1 is a proto-oncogene whose transforming effect is primarily manifested due to miss-expression and gain of function mutations, such as the FIG-ROS fusion, which targets the intracellular region of ROS1 to the golgi apparatus in glioblastomas (Charest et al., 2003, 2003), and the recently identified SLC34A2-ROS and CD74-ROS fusions in lung carcinomas (Rikova et al., 2007). With currently available techniques, construction of molecules that mimic ROS1 activating mutations in *C. elegans* are easily attainable, and would be a potentially informative approach to increase our understanding of the biology of this oncogene.

The receptor tyrosine kinase ROL-3 is at a hub of *C. elegans* hypodermal development, and provides an essential developmental role in epithelial development. Lack of ROL-3's function to promote seam cell elongation potentially uncouples crosstalk between specialized epithelial tissues, leading to severe pleiotropic defects in epithelial development, cuticle formation and molting. Further investigation will elucidate the precise molecular mechanisms involved. Finally, research of ROL-3 function in *C. elegans* development has potential utility as a model system for investigation of both the oncogenic and endogenous function of the Human proto-oncogene ROS1.

MATERIALS AND METHODS

Maintenance and handling of *C. elegans* were performed as previously described (Brenner, 1974). Worms were cultured at 20°, unless otherwise stated. Bristol N2 was used as Wild Type.

Mutant strains: LGI: *dpy-5(e907)*, LGII: *srap-1(sDp33)*, LGIV: *bcc-1(tm3821), him-8(e1489)*, LGV:

rol-3(e202, e754, s126, s422, s1030, s1040ts, s1409, s1519, tm3908)

Transgenic arrays: *kaIs12 (COL-19::GFP), syIs78* (AJM-1::GFP)*, wIs78* (SCM::GFP; AJM-1::GFP)*, gaIs233 (elt-5::HIS-24::mCherry)*, *ouEx123* (AJM-1::mCherry, RNT-1::GFP)*, oxIs12 (unc-47::GFP), sEX1594 (rol-3::GFP), sEx2692* (ROL-3::GFP), *sEx1743 (bcc-1::GFP), sEx2127 (srap-1::GFP), sEx2150* (SRAP-1::GFP).

Fosmid selection and Preparation: *C. elegans* genomic fosmid clones containing *C16D9.2 (rol-3)* and *T06D8.1 (srap-1)* were identified via the web-based searching facility at the Michael Smith Genome Sciences Centre (http://elegans.bcgsc.bc.ca/perl/fosmid/CloneSearch). Fosmid DNAs were isolated as previously described (Dolphin and Hope, 2006).

Generation of fosmid subclones: A subclone of fosmid WRM067dH05 was generated by restriction enzyme digestion of 1 mg of fosmid with 10U *Spe*I (NEB) followed by gel extraction of the 18 kb fragment predicted to contain C16D9.2. The 18 kb fragment was subsequently injected at 10 μg/ml together with 80 μg/ml of *pCeh361* and 10 μg/ml of *pTG96* of into *dpy-5* animals.

Generation of fluorescent reporters by PCR stitching: To generate transcriptional reporters putative promoters regions were amplified by PCR and fused to GFP using PCR stitching as previously described (Hobert, 2002). Primer sequences for *rol-3::GFP*, *bcc-1::GFP*, *srap-1::GFP* are available upon request.

Generation of translational fluorescent reporters by recombineering: Translational fluorescent reporters were constructed by recombineering as previously described (Tursun et al., 2009). Primer sequences for *ROL-3::GFP* and *SRAP-1::GFP* are available upon request.

Generation of transgenic strains: Transgenic animals were generated as previously described (Hunt-Newbury et al., 2007). DNA constructs were co-injected at an initial concentration of 10 μg/ml with 80μg/ml of *pCeh361* (a *dpy-5* rescuing construct; Thacker et al., 2006) and 10 μg/ml of *pTG96* (a *myo-2* promoter green fluorescent protein reporter plasmid, which has strong expression in the pharynx) (Gu et al., 1998). To generate transgenic animals containing *C16D9.2*, a fosmid DNA concentration of between 1 and 2 μg/ml was necessary. To confirm that each transgenic line contained fosmid DNA, a 600 bp PCR product targeting the fosmid vector sequence was amplified (Primer sequences available upon request).

Rescue of *rol-3* lethal alleles: Hermaphrodites carrying a transgenic array containing the wild type *rol-3* ORF and the *myo-2::GFP* pharyngeal fluorescent reporter mated to N2 males. *myo-2::GFP* positive males were isolated from the F1 progeny and mated to *dpy-18(e364)/eT1(III)*; *rol-3(x)*(where x is any lethal allele of *rol-3*), *unc-46(e177)/eT1(V)* hermaphrodites. Rescue of *rol-3* was confirmed by the presence of viable *myo-2::GFP* positive *Unc* animals in the F2. *rol-3(s833)* is linked to *dpy-11(e224)*, in this case rescue was assayed by the presence of viable *myo-2::GFP* positive F2 *Dpy* animals.

Sequencing of mutant alleles: 50 animals of the appropriate genotype were picked into 50μl of lysis buffer (50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.45% Tween-20, 0.01% Gelatin, 0.1 mg/ml Proteinase K) and freeze cracked in liquid nitrogen. Samples were lysed and treated with proteinase K, (1 h at 60° followed by 15 min at 95°) and 1μl subsequently used in a 25 μl PCR reaction with appropriate primers (available on request). Samples were sent for sequencing at Macrogen (https://dna.macrogen.com/english/login/join.jsp).

Lectin staining of *rol-3(s1040)* animals: Fluorescein isothiocyanate (FITC) coupled wheat germ agglutinin (WGA; Sigma; Cat. No. L4895) was used to test for exposure of specific antigens on the surface of *rol-3(s1040)* animals raised at 20°. Mixed stage worm cultures were washed in sterile M9 buffer three times followed by incubation in M9 buffer for another hour to remove residual bacteria from the gut and body surface. Worms were incubated in 100 μl FITC conjugated WGA (20–25 μg/mL) for 1 h, washed in 1 ml M9 buffer four times before being mounted on slides for observation with Nomarski and fluorescent microscopy.

Microscopy and Image Processing: Analysis of mutant and GFP transgenic animals was performed using a ZEISS Stemi SVC11 dissecting microscope with GFP filters and a ZEISS Axioskop with 10×/0.25, 40×, 0.65 and 60×/0.85 Objective Lenses. All pictures were taken using QCapture software (QImaging) with a QIMAGING digital camera mounted on a ZEISS Axioskop. Images were processed using Photoshop CS5 (Adobe).

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