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TORC2 signaling antagonizes SKN-1 to induce *C. elegans* mesendodermal embryonic development

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Abstract

The evolutionarily conserved *target of rapamycin* (TOR) kinase controls fundamental metabolic processes to support cell and tissue growth. TOR functions within the context of two distinct complexes, TORC1 and TORC2. TORC2, with its specific component Rictor, has been recently implicated in aging and regulation of growth and metabolism. Here, we identify *rict-1/Rictor* as a regulator of embryonic development in *C. elegans*. The transcription factor *skn-1* establishes development of the mesendoderm in embryos, and is required for cellular homeostasis and longevity in adults. Loss of maternal *skn-1* function leads to misspecification of the mesendodermal precursor and failure to form intestine and pharynx. We found that genetic inactivation of *rict-1* suppressed *skn-1*-associated lethality by restoring mesendodermal specification in *skn-1* deficient embryos. Inactivation of other TORC2 but not TORC1 components also partially rescued *skn-1* embryonic lethality. The SGK-1 kinase mediated these functions downstream of *rict-1*/TORC2, as a *sgk-1* gain-of-function mutant suppressed the *rict-1* mutant phenotype. These data indicate that TORC2 and SGK-1 antagonize SKN-1 during embryonic development.

Keywords

TOR signaling; RICTOR; SGK-1; SKN-1; Development; *C. elegans*

Introduction

The mammalian *target of rapamycin* (TOR) kinase is highly conserved in eukaryotes and integrates nutrient and anabolic signals to promote growth (Laplante and Sabatini, 2009; Wullschleger et al., 2006; Zoncu et al., 2011). TOR participates in two biochemically and functionally distinct kinase complexes, TORC1 and TORC2. TORC1 consists of TOR, Raptor (regulatory associated protein of mTOR), and LST8 (lethal with Sec13 protein8).

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Appendix A. Supporting information Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.08.011.

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Growth signals, nutrients, oxygen, and energy levels have been shown to activate TORC1. TORC1 regulates a set of well-characterized substrates to promote growth-related processes including protein synthesis, ribosome biosynthesis, and transcription, and to inhibit autophagy (reviewed in Loewith and Hall, 2011).

TORC2 includes the conserved proteins Rictor (rapamycin-insensitive companion of TOR), LST8, and Sin1 (stress activated protein kinase interacting protein 1), along with TOR. In contrast to TORC1, the upstream inputs and downstream effectors of TORC2 are poorly understood. Recent studies demonstrated that growth signals and association with ribosomes activate TORC2 (Oh et al., 2010; Zinzalla et al., 2011). TORC2 phosphorylates AGC kinases such as Akt and SGK (serum- and glucocorticoid-induced protein kinase) to activate downstream signaling (Zoncu et al., 2011). Phosphorylation of Akt at the hydrophobic site by TORC2 is important for its functions in cellular processes including cell proliferation and survival (Hresko and Mueckler, 2005; Sarbassov et al., 2005). Similarly to Akt, SGK1 is phosphorylated at its hydrophobic motif site by TORC2, which thereby regulates its activity (Garcia-Martinez and Alessi, 2008).

Rictor is required for TORC2 integrity and substrate specificity. Previous studies have established a conserved role for TORC2 in cell growth and metabolism (Cybulski et al., 2009; Hietakangas and Cohen, 2007; Shiota et al., 2006). Knockdown of *Rictor/rict-1* in *Caenorhabditis elegans* resulted in an overall reduction of body size, developmental delay, and defects in fat metabolism (Jones et al., 2009; Soukas et al., 2009). Interestingly, these effects of *rict-1* signaling were largely mediated through SGK-1, rather than AKT-1/2 in *C. elegans*. Moreover, genetic studies in *C. elegans* have shown that inhibition of *rict-1* during adulthood confers longevity (Robida-Stubbs et al., 2012). Importantly, the transcription factor *skn-1* was required for lifespan to be increased by *rict-1* knockdown. SKN-1 and its mammalian ortholog Nrf have conserved functions in stress detoxification, and contribute to longevity (Sykiotis and Bohmann, 2010). SKN-1/Nrf orchestrates gene expression programs involved in stress resistance, longevity, protein homeostasis, and metabolism (Oliveira et al., 2009; Paek et al., 2012; Wang et al., 2010). The AKT and SGK-1 kinases phosphorylate SKN-1 and thereby prevent its accumulation in intestinal nuclei and inhibit its functions (Tullet et al., 2008).

SKN-1 was originally identified as an essential regulator of mesendodermal development during the earliest stages of *C. elegans* embryogenesis (Bowerman et al., 1992). Maternally expressed SKN-1 specifies the fate of the EMS blastomere. The E cell defines the endoderm comprising the intestine, while the MS cell gives rise to the mesoderm including the posterior portion of the pharynx, body muscle cells, and coelomocytes (for review see Maduro, 2010). Loss of *skn-1* leads to a complete lack of MS-derived tissues and to most, but not all, embryos also lacking intestinal cells (Bowerman et al., 1992,1993). SKN-1 directly induces expression of the transcription factors MED-1 and MED-2, which are required for mesendodermal differentiation of the EMS lineage (Coroian et al., 2006; Maduro et al., 2001). In MS, the MEDs activate the T-box transcription factor *tbx-35* and initiate a gene network for mesodermal specification (Broitman-Maduro et al., 2006). Specification of the E fate results from activation of two GATA factor-encoding genes, *end-1* and *end-3*, by the MEDs and SKN-1 (Maduro et al., 2005; Owraghi et al., 2010; Zhu et al., 1997). These pathways finally converge on the activation of the GATA factor *elt-2*, which is essential for E lineage development and maintenance (Fukushige et al., 1999).

The regulatory cascade initiated by SKN-1 collaborates with the Wnt/β-catenin asymmetry pathway to distinguish MS and E identity. EMS receives an induction signal from its posterior sister P2 that results in differential localization and activity of POP-1, a TCF/Lef-related factor within MS and E. In the MS blastomere, POP-1 represses the endoderm-

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specific gene program allowing mesoderm development to occur. Inside the E cell, this repression is relieved and POP-1 is converted to an activator of the endoderm gene expression network (Lin et al., 1995; Maduro and Rothman, 2002; Rocheleau et al., 1997; Thorpe et al., 1997).

Given that SKN-1 seemed to be required for effects of TORC2 on longevity, we considered whether TORC2 might influence SKN-1 functions more broadly. Here, we define a new function for *C. elegans* TORC2 during early embryonic stages. We show that *rict-1* mutation decreased the embryonic lethality associated with loss of *skn-1* function by allowing mesendodermal development to be reestablished in a proportion of *skn-1* deficient embryos. A gain-of-function mutation in *sgk-1* suppressed these *rict-1* developmental effects, indicating that *sgk-1* is a primary effector of *rict-1* activity in this context. In contrast to TORC2, TORC1 did not influence SKN-1 activity in the embryo. Our results define TORC2-SGK-1 as a pathway that may broadly influence SKN-1 functions.

Results

Loss of rict-1 rescues lethality of skn-1 deficient embryos

The transcription factor SKN-1 is well established as an initiator of mesendodermal development in embryos, and controls oxidative stress response. Since TORC2 has been recently implicated in the regulation of SKN-1 dependent longevity, we wondered if it interferes with SKN-1 functions more generally and might be involved in embryonic development. Rictor (RICT-1) is an essential component of TORC2. We initiated our study by analyzing the effects of *rict-1* mutation on *skn-1*-dependent developmental processes. The *rict-1* alleles *mg451* and *ft7* contain early stop mutations, and are likely to be strong loss-of-function and null alleles, respectively (Jones et al., 2009; Soukas et al., 2009). Embryos from *skn-1* deficient mothers undergo developmental arrest, lack pharynx and endoderm, and die (Bowerman et al., 1992). First, we used RNAi feeding to inactivate maternal *skn-1* function and to sensitize our ability to detect genetic interactions. We scored the portion of the *skn-1*(RNAi) progeny that hatched and progressed past the first larval stage (L1). Consistent with the crucial role of *skn-1* during development, *skn-1*(RNAi) resulted in 0.4% viable embryos. Surprisingly, we found that combining *skn-1*(RNAi) with mutational *rict-1* inactivation strongly increased the viability of the progeny: 8.2 and 14.1% of eggs produced by *rict-1*(*ft7*) and *rict-1*(*mg451*) mutants, respectively, fed with *skn-1*(RNAi) give rise to viable offspring (Fig. 1A and Table 1). Here, *rict-1*(*mg451*) and *rict-1*(*ft7*) survival was not statistically different. We also injected dsRNA corresponding to the *skn-1* gene into the gonad of *rict-1(mg451)* mutants and wild-type worms and again observed that about 6% of *rict-1*(*mg451*);*skn-1*(RNAi) embryos hatched while 100% of *skn-1*(RNAi) embryos arrested development (data not shown). To test whether *rict-1* specifically influenced *skn-1* function and rule out that inactivation of *rict-1* interferes with sensitivity to dsRNA per se, we tested two embryonic-lethal genes that to our knowledge are not defective in functions related to *skn-1*: *let-423* and *cdk-2*. The complete embryonic lethality that was associated with RNAi-mediated knockdown of these genes was not altered in *rict-1* mutants (data not shown).

Next, we generated *rict-1*;*skn-1* double mutants and analyzed the fraction of survivors. The *skn-1* mutations *zu67* and *zu135* introduce premature stop codons and are predicted to be strong loss-of-function alleles based on the removal of the conserved DNA binding domain (Supplementary Fig. S1). Adult hermaphrodites homozygous for mutations in *skn-1* produced no viable offspring, while double knockout of *rict-1*(*mg451*) and *skn-1*(*zu67*) significantly suppressed embryonic lethality. 3.9% of embryos produced by *rict-1*;*skn-1* double mutants were viable and hatched (Fig. 1B). This finding was confirmed by several alleles for *rict-1* and *skn-1* (Table 2). Thus, *rict-1* inactivation suppressed the embryonic

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lethality caused by *skn-1* deficiency. Interestingly, *rict-1*;*skn-1* mutant embryos that hatched developed into reproductive adults. They did not show obvious morphological defects and even produced progeny, although they grew slower and were smaller in body size than was characteristic of *rict-1* mutants (Supplementary Fig. S2). The observation that the embryonic lethality associated with *skn-1* can be partially suppressed by inhibition of *rict-1* suggests that *rict-1* and TORC2 antagonize *skn-1* functions during development.

rict-1 acts in a pathway with sgk-1 to effect skn-1 embryo survival

TORC2 has been reported to activate several AGC family kinases, including AKT and the serum- and glucocorticoid-induced kinase SGK (Garcia-Martinez and Alessi, 2008; Sarbassov et al., 2005). To investigate the mechanisms through which *rict-1* regulates embryonic development, we first focused on Akt signaling. We analyzed the effect of *akt-1* and *akt-2* knockdown on *skn-1*(−) embryo survival. However, inactivation of *akt-1* or *akt-2* did not restore viability of *skn-1*(RNAi) embryos ((0.6% viable embryos in *akt-1*(*ok525*);*skn-1*(RNAi) and 0.5% in *akt-2*(*ok393*);*skn-1*(RNAi) compared to 8.8% in *rict-1*(*mg451*);*skn-1*(RNAi)) (Table 1). We conclude that the increased viability of *rict-1*;*skn-1* deficient embryos is not a consequence of compromised AKT signaling.

In *C. elegans*, *rict-1* regulates growth, larval development, and metabolism largely through *sgk-1* (Jones et al., 2009; Soukas et al., 2009). To evaluate the possible role of *sgk-1* in embryonic development we analyzed the effect of *sgk-1* knockdown on *skn-1*(−) embryo survival. The *sgk-1*(*ok538*) deletion removes most of the region encoding the kinase domain, and is likely a null allele (Hertweck et al., 2004). We found that *sgk-1* mutants rescued the lethality of *skn-1* deficient embryos: 10.7% of *sgk-1*(*ok538*);*skn-1*(RNAi) embryos were viable compared to 0.3% *skn-1*(RNAi) (Fig. 1C and Table 1). Moreover, *skn-1*(*zu67*);*sgk-1*(*ok538*) double mutants produced 1.8% viable embryos, while all embryos from *skn-1*(*zu67*) mutant underwent developmental arrest and died (Table 2). Hence, loss of *sgk-1* function mimics *rict-1*, in that it partially rescued *skn-1*(−) embryonic lethality, suggesting a genetic relationship.

To assess whether *rict-1* and *sgk-1* define a genetic pathway in the embryo, we created *rict-1*(*mg451*);*sgk-1*(*ok538*) double mutants. 7.8% of *rict-1*;*sgk-1* double mutants fed with *skn-1*(RNAi) gave viable progeny (Fig. 1D and Table 1). There was no significant difference in fraction of survivors compared to either single mutant (9.3% for *rict-1*(*mg451*);*skn-1*(RNAi) and 6.8% for *sgk-1*(*ok538*);*skn-1*(RNAi)). These results suggest that *rict-1* and *sgk-1* act in the same pathway.

This hypothesis was further strengthened by the analysis of a *sgk-1* gain-of-function mutant. If *sgk-1* acts downstream of *rict-1* to regulate *skn-1* embryonic development then a gain-of-function mutation in *sgk-1* should suppress the viability of *rict-1*(−);*skn-1*(−). The *sgk-1*(*ft15*) gain-of-function mutant has been previously shown to suppress many phenotypes associated with *rict-1* (Jones et al., 2009). We indeed found that *sgk-1*(*ft15*) greatly suppressed the fraction of viable embryos of *rict-1*;*skn-1*(RNAi), 1.7% of *rict-1*(*mg451*);*sgk-1*(*ft15*);*skn-1*(RNAi) progeny were viable, compared to 11.5% in *rict-1*(*mg451*);*skn-1*(RNAi) (Fig. 1E and Table 1). *sgk-1*(*ft15*) mutants displayed normal development and broodsize and did not influence *skn-1*(−) embryo survival. Taken together, these obser vations indicate that *rict-1* functions through *sgk-1* to regulate embryonic development mediated by *skn-1*.

TORC1 inhibition cannot suppress skn-1 embryonic lethality

TOR exists in two distinct complexes: association with RICT-1/CeRictor defines TORC2, while DAF-15/CeRaptor is the essential component of TORC1. We wondered whether the

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effect of *rict-1* inhibition on embryonic development might be phenocopied by components of TORC1.

The *rheb-1* GTPase is the key activator of TORC1 (Honjoh et al., 2009; Long et al., 2005; Yang and Guan, 2007). We found that knockdown of *rheb-1* by RNAi did not suppress lethality of *skn-1*(*zu67*) mutant embryos. Although *rheb-1*(RNAi) efficiently reduced the RHEB-1 levels as evidenced by greatly abolished expression of a *rheb-1*::*GFP* reporter construct (data not shown), fewer than 0.4% of *skn-1*(*zu67*);*rheb-1*(RNAi) embryos were viable, while *sgk-1*(RNAi) significantly rescued the lethality of *skn-1* deficient embryos (Fig. 2A and Table 3). Likewise, simultaneous RNAi-mediated knockdown of *rheb-1* and *skn-1* did not increase the fraction of viable embryos as compared to *skn-1*(RNAi) alone (Table 3). *daf-15* encodes the *C. elegans* homolog of Raptor, and is essential for TORC1 activity. We found that knockdown of *daf-15* failed to rescue embryonic lethality of *skn-1* mutants (Fig. 2A), while *daf-15*(RNAi) strongly reduced *daf-15* activity and caused a dauer-like larval arrest (data not shown) (Jia et al., 2004). Consistently, combined inhibition of *daf-15* and *skn-1* by RNAi did not promote embryonic viability (Table 3). The TOR kinase *let-363* is at the center of both TOR complexes and therefore affects function of TORC1 and TORC2. As would be predicted, RNAi against *let-363* suppressed the lethality of *skn-1* mutants. 1.1% of eggs produced by *skn-1*(*zu67*);*let-363*(RNAi) gave rise to viable offspring compared to 0% of *skn-1*(*zu67*) (Fig. 2A and Table 3).

TORC1 plays an essential role during development. Consistent with published findings, inactivation of TORC1-specific genes by RNAi starting from L1 caused a dauer-like larval arrest of the F1 progeny (Jia et al., 2004; Long et al., 2002). Similarly, homozygous TORC1 mutants arrest larval development. To exclude that complete TORC1 inactivation might mask a rescue effect on *skn-1* by interfering with larval development, we analyzed heterozygous TORC1 component mutants. Previous studies have shown that heterozygous *daf-15* mutants display no obvious developmental defects but show significantly extended lifespan, indicating that loss of gene dose might decrease *daf-15* activity enough to be effective (Jia et al., 2004). Nevertheless, we found that *daf-15*(*m81*) heterozygousity did not suppress *skn-1*(RNAi) embryonic lethality (Supplementary Fig. S3 and Table 3). Consistent with the results on *let-363*(RNAi), heterozygous *let-363* mutants slightly rescued the lethality of *skn-1* deficient embryos, 1.9% of *let-363*(*h111*)/+;*skn-1*(RNAi) embryos were viable compared to 0.2% *skn-1*(RNAi) (Supplementary Fig. S3 and Table 3).

Taken together these results indicate that inhibition of TORC1-specific components *rheb-1* and *daf-15/Raptor* cannot suppress lethality of *skn-1* deficient embryos, suggesting that TORC1 does not influence *skn-1*-mediated regulation of embryonic development. Since inhibition of the TOR kinase *let-363* would eliminate TORC1 as well as TORC2 knockdown of *let-363*/TOR might at least partially promotes viability of *skn-1* embryos.

Inhibition of TORC2 prevents *skn-1* embryonic lethality

The TORC2 complex includes the LST8 and Sin1 proteins, in addition to Rictor and TOR. To investigate further whether TORC2 regulates *skn-1* embryonic functions, we examined the role of these two proteins. *sinh-1* encodes the *C. elegans* ortholog of mammalian Sin1, which plays a key role in Akt phosphorylation and signaling (Yang et al., 2006). Inactivation of *sinh-1* by RNAi only slightly rescued embryonic lethality of *skn-1* mutants (Fig. 2B and Table 3). However, combined knockdown of *sinh-1* and *skn-1* by RNAi significantly suppressed *skn-1* embryonic lethality, 4.1% of *sinh-1*(RNAi);*skn-1*(RNAi) embryos were viable compared to 0.3% of *skn-1*(RNAi) (Table 3).

Next, we analyzed the effect of *lst-8*. LST8 is present in both TOR complexes, but recent data indicate that mLST8 is critical for TORC2 function (Guertin et al., 2006; Wang et al.,

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2012). We found that loss of *lst-8* activity by RNAi resulted in increased fraction of 12.3% viable *skn-1*(*zu67*) embryos (Fig. 2B and Table 3). Surprisingly the effect of *lst-8*(RNAi) on *skn-1*(−) viability was significantly stronger than *rict-1* or *sgk-1*.

Taken together, inhibition of TORC2-specific components, *rict-1*, *lst-8*, and *sinh-1* can partially restore embryonic viability of *skn-1* deficient embryos. These findings indicate new physiological roles of TORC2 in the regulation of embryonic development mediated by *skn-1*.

Inactivation of rict-1 promotes mesodermal specification

SKN-1 activates a complex gene network that specifies the development of endoderm (intestine) and part of the mesoderm (including pharynx and body muscle). Embryos from *skn-1* deficient mothers arrest development and fail completely to specify the pharynx, while endoderm is absent in approximately 70% of the embryos (Bowerman et al., 1992, 1993). In the EMS blastomere SKN-1 directly activates expression of the two transcription factors, *med-1* and *med-2* (Coroian et al., 2006; Maduro et al., 2001). To promote specification of the endoderm, SKN-1 and the MEDs initiate a short signaling cascade by activating the GATA factor genes *end-1* and *end-3*. Either END transcription factor can then activate the GATA factor *elt-2*, which is a key regulator of intestinal development (Fukushige et al., 1998; Goszczynski and McGhee, 2005; Maduro et al., 2007). The fate of the mesodermal precursor cell MS is specified primarily by activation of the T-box transcription factor *tbx-35* by the MEDs (Broitman-Maduro et al., 2006, 2009). TBX-35 then regulates tissue-specific factors for pharynx and muscle development (Fig. 3A).

Given the impact of TORC2 on regulation of *skn-1* embryonic development, we wondered whether *rict-1* interferes with the gene network for mesendodermal specification. First, we assessed the production of MS-derived cell types. The MS blastomere generates diverse cell types, including pharyngeal cells. *ceh-22* is expressed exclusively in the pharyngeal muscles, and together with *pha-4* it is the earliest known marker of pharyngeal muscle differentiation (Okkema and Fire, 1994). To score the production of pharyngeal muscle cells we used a chromosomally integrated *ceh-22*::GFP reporter. As expected, *skn-1* deficient embryos did not show any significant expression of *ceh-22*::GFP. In contrast, we found that 46% of *rict-1*(*mg451*);*skn-1*(RNAi) embryos produced pharyngeal muscle cells (Fig. 3B, D and F). *rict-1* mutants alone showed normal pharynx development and *ceh-22*::GFP expression similar to wild-type (Fig. 3B, C and E). Interestingly, three times more *rict-1*;*skn-1* embryos displayed induction of pharyngeal development (46%) while only one third completed development, hatched and were viable. The majority of *rict-1*;*skn-1* embryos arrested at varying stages. Of note, we did not detect ectopic *ceh-22*::GFP expression in *rict-1*;*skn-1* embryos. Hence, inactivation of *rict-1* seems to restore pharyngeal specification to a greater extent than is apparent based upon scoring for viability.

E-derived tissues are made in rict-1;skn-1 Embryos

The principal target of the E-specification gene network initiated by SKN-1 is the transcription factor *elt-2* (Fukushige et al., 1998). ELT-2 is required for embryonic gut development, and maintenance of the gut throughout larval development and adulthood (Fukushige et al., 1998; McGhee et al., 2007, 2009). To assess endoderm development, we introduced a chromosomally integrated *elt-2*::GFP reporter into *rict-1* mutants. Expression of *elt-2*::GFP was detected in 55% of *rict-1*(*mg451*);*skn-1*(RNAi) embryos, while inactivation of *skn-1* alone resulted in 29% of embryos with intestinal cells (Fig. 4B, D, and E). *rict-1* mutants expressed *elt-2*::GFP in almost 100% of embryos similarly to wild-type (Fig. 4A, C, and E). This result suggests that inhibition of *rict-1* partially rescues impaired gut formation in *skn-1* deficient embryos. The observation that a fraction of *skn-1* deficient

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embryos still generates intestinal cells is in line with the literature (Bowerman et al., 1992; Maduro et al., 2007) and might be the consequence of endoderm specification from a number of parallel activities.

Next, we wondered if the induction of endoderm development by loss of *rict-1* might reflect changes in the number of gut cells. We therefore quantified the number of gut cells using the integrated *elt-2*::GFP reporter, as it has been performed in previous studies (Kostic and Roy, 2002; Maduro et al., 2007). The E cell normally divides 4–5 times to produce 20 intestinal cells by the end of embryogenesis (Sulston et al., 1983). As expected, wild-type embryos produced an average of 20 *elt-2* expressing cells with little variation (Supplementary Fig. S4). We found that *rict-1* mutant embryos generate a nearly identical mean number of endodermal cells as detected in wild-type (18.9±0.2), although with a slightly higher variance. The increased variance is likely to have resulted from the delayed development in *rict-1* mutants (Soukas et al., 2009) rather than differences in gut cell number. In *rict-1* mutant embryos we did not detect *elt-2*::GFP expression in more than 20 cells. Among *skn-1*(RNAi) embryos that still made endoderm, the average number of gut cells was dramatically reduced to 8.9±0.5, with a range of 2–18 gut cells. *rict-1*(*mg451*);*skn-1*(RNAi) embryos showed a higher mean number of gut cells (15.1±0.5, range of 4–20) and a higher portion of embryos (10%) with a normal number of gut cells compared to *skn-1*(RNAi) (Supplementary Fig. S4). We conclude therefore that loss of *rict-1* function can promote normal development of the endoderm in *skn-1* embryos.

Loss of rict-1 induces med-1 in embryos lacking skn-1

Our finding that E- and MS-specific genes are expressed in *rict-1*;*skn-1* embryos supports the hypothesis that the canonical pathway for E and MS specifications is active. Two transcription factors, *med-1* and *med-2*, are immediately activated by SKN-1 in EMS and essential for endomesodermal development (Maduro et al., 2001, 2007). To further analyze whether intestinal and pharyngeal cells made in *rict-1*;*skn-1* embryos result from normal EMS specification, we introduced the translational fusion reporter *med-1*::GFP. In wild-type embryos, *med-1*::GFP expression is first detectable in EMS at the six-cell stage, and this expression is transient and becomes weaker in E and MS descendents (Maduro et al., 2001, 2007). Inactivation of *skn-1*(RNAi) alone resulted in nearly complete abrogation of *med-1* expression (0.8%) while expression of *med-1*::GFP was observed in 9.7% of *rict-1*(*mg451*);*skn-1*(RNAi) embryos (Fig. 5). Together, these results show that inactivation of *rict-1* induces mesendodermal specification in *skn-1* deficient embryos involving activation of the canonical genetic network including *med-1*.

sknr-1 cannot compensate for loss of skn-1 function in the embryo

We have established above that TORC2 inhibits SKN-1 functions in the embryo. The simplest explanation is that TORC2 acts on SKN-1 itself, but this would require that residual SKN-1 function be present in the *skn-1* mutants we analyzed. Alternatively, *rict-1* might affect processes in parallel to or downstream of SKN-1. First, we evaluated the possibility of residual SKN-1 functions in *skn-1*(*zu67*) mutants. Low levels of residual SKN-1 protein might derive from perdurance of the maternal gene product in the *skn-1* (−/−) animal, or from read-through. *rict-1*(*mg451*);*skn-1*(*zu67*) homozygous animals generated about 4% viable embryos (Fig. 1B and Table 2). These “rescued” offspring themselves produced viable rescued offspring at a similar rate (not shown), arguing against perdurance of maternal SKN-1. Supposing that SKN-1 still bears residual function in *skn-1* mutants, we hypothesized that knockdown of *skn-1* by RNAi might suppress rescue of lethality in *rict-1*;*skn-1* mutants. Using *skn-1*(RNAi), the fraction of survivors in *rict-1*(*mg451*);*skn-1*(*zu67*) was reduced to 2.8%, compared to 5.2% survivors in

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rict-1;*skn-1*;*control*(RNAi) (Table 4). Hence, residual SKN-1 function might at least partially contribute to the viability of *rict-1*;*skn-1* embryos.

We considered whether TORC2 and SGK-1 might regulate *skn-1* transcription, providing a possible mechanism for restoration of viability. When we performed quantitative PCR to measure endogenous amounts of *skn-1* mRNA, we found that *skn-1* expression was not changed in *rict-1* and *sgk-1* mutants compared to wild-type (Supplementary Fig. S5). The levels of *skn-1* mRNA decreased in *skn-1*(*zu67*) and *skn-1*(*tm3411*) mutants consistent with a previous study (Tullet et al., 2008), and we observed similar *skn-1* expression in *rict-1*;*skn-1* double mutants. Hence, *rict-1* and *sgk-1* do not affect SKN-1 functions by modulating the abundance of *skn-1* transcription.

We next investigated whether increased SKN-1 protein stability might influence *rict-1*;*skn-1* embryonic viability. It was reported previously that WDR-23 represses SKN-1 activity by targeting SKN-1 for degradation (Choe et al., 2009; Hasegawa and Miwa, 2010). Accordingly, loss of *wdr-23* increased SKN-1 protein levels and activity. Here, we tested whether knockdown of *wdr-23* affects *rict-1*;*skn-1* embryonic viability. However, *wdr-23*(RNAi) did not alter the fraction of viable *rict-1*;*skn-1* embryos (Table 4). This suggests that *wdr-23* might not influence SKN-1 activity in the embryo, or that increased levels of embryonic SKN-1 per se are not sufficient to enhance rescue of the embryonic *skn-1* phenotype.

To address further whether residual SKN-1 might be required for TORC2 embryonic functions, we tested the effect of a *skn-1* allele that is predicted to be null: *tm3411* bears a deletion that should essentially destroy the DNA binding capacity of SKN-1 (Blackwell et al., 1994) (Supplementary Fig. S1). Interestingly, we still observed 2.4% viable embryos in *rict-1*(*mg451*);*skn-1*(*tm3411*), while all embryos of *skn-1*(*tm3411*) underwent developmental arrest and died (Table 4). This result suggests that the increased viability of *rict-1*;*skn-1* mutants might not be solely the consequence of residual SKN-1 activity, and argues towards involvement of an alternative downstream process that works together with SKN-1.

Following the hypothesis that *rict-1* could exert its functions independently of *skn-1* and modulate another factor we considered the possibility that this might involve SKNR-1, a protein that is closely related to SKN-1 but so far has no known function (Blackwell et al., 1994; Bowerman et al., 1992) (Supplementary Fig. S1). To assess if *sknr-1* can compensate for loss of *skn-1* function and account for viability in *rict-1*;*skn-1* embryos we created *rict-1*;*skn-1*; *sknr-1* triple mutants. We found that mutation of *sknr-1* did not affect *rict-1*;*skn-1* viability: there was no difference in the fraction of survivors between *rict-1*(*mg451*);*skn-1*(*zu135*) mutants and *rict-1*;*skn-1*;*sknr-1*(*tm2386*) (Table 4). *sknr-1* mutants displayed no obvious defects in development. We also tested a different *sknr-1* allele, *ok1216*. Again, *sknr-1*(*ok1216*) did not change the viability of *rict-1*;*skn-1* mutant embryos (Table 4). These observations indicate that *sknr-1* does not contribute to the rescue of embryonic lethality by *rict-1*. Together, the data suggest that the embryonic functions of RICT-1/TORC2 involve SGK-1 and SKN-1, but also that RICT-1/TORC2 might regulate an alternative, as yet undefined process that acts in parallel to SKN-1 in the embryo.

Discussion

We have identified *rict-1/CeRictor* as a new regulator of embryonic development in *C. elegans*. Our findings revealed that mutation of *rict-1* re-established mesendodermal specification in *skn-1* deficient worms, and thereby suppressed *skn-1*-associated lethality. Moreover, inactivation of *let-363/CeTOR*, *lst-8*, and *sinh-1*, but not *rheb-1* and *daf-15*/Raptor partially rescued *skn-1* embryonic lethality. Thus, TORC2 but not TORC1

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antagonizes *skn-1* during embryonic development. Importantly, *sgk-1* mediated these functions downstream of *rict-1*/TORC2 as the *rict-1* mutant phenotype was phenocopied by loss of *sgk-1* function, and suppressed by a *sgk-1* gain-of-function mutant.

Inactivation of rict-1/TORC2 promotes skn-1 developmental processes

rict-1 deficient worms display pleiotropic phenotypes, including reduced growth and body size, metabolic changes of the fat content, and increased lifespan compared to wild-type (Jones et al., 2009; Robida-Stubbs et al., 2012; Soukas et al., 2009). Our previous analyses suggested that TORC2 opposed *skn-1* to regulate aging (Robida-Stubbs et al., 2012). We now describe a function of *rict-1/TORC2* in *skn-1* dependent developmental processes. *skn-1* mutant embryos lack mesodermal tissue, and most but not all embryos also fail to specify endoderm (Bowerman et al., 1992; Maduro et al., 2007; our own data). While essentially all *skn-1* deficient embryos are dead, loss of *rict-1* restored viability to a small but reproducibly detectable fraction of the embryos. We observed that a fraction of *rict-1*;*skn-1*-deficient embryos generated a complete pharynx and intestine (based upon morphology and staining of *ceh-22* and *elt-2*). Furthermore, expression of *med-1*, which is a direct target of *skn-1* and correlates with *skn-1* function (Maduro et al., 2001, 2007), was induced by *rict-1* deficiency indicating activation of the canonical SKN-1 network for mesendodermal specification by EMS. Interestingly, inactivation of *rict-1* restored not only formation of the posterior, MS-derived portion of the pharynx but also of the anterior pharynx in *skn-1* deficient embryos. The anterior portion of the pharynx is specified by cell-cell interaction between MS and ABa descendants (Fig. 3A). A *skn-1* dependent signal is expressed in the MS cell that triggers anterior pharynx development in ABa. The identity of this factor remains unknown, though it is likely to be a DSL (Delta/Serrate/Lag) family ligand that induces anterior pharynx development through the GLP-1/Notch receptor (Bowerman et al., 1992; Mango et al., 1994; Priess et al., 1987). Thus, at least two distinct SKN-1 targets are restored to their correct spatiotemporal expression in *rict-1* mutant embryos: *med-1/2* assigning mesendodermal specification as well as Delta-like factor signaling from MS to ABa.

Our data suggest that genetic interference with *rict-1/TORC2* might release *skn-1* function from inhibition, and promote developmental processes. Consistent with the hypothesis that *rict-1* inactivation enhances SKN-1 activity, knockdown of residual *skn-1* functions by RNAi in *rict-1*(*mg451*);*skn-1*(*zu67*) mutants significantly abolished embryonic viability, albeit the effect was not complete. Of note, the *skn-1* alleles *zu67* and *zu135* show incomplete penetrance of the mesendodermal differentiation phenotype and may represent strong loss-of-function rather than true null alleles (Bowerman et al., 1992; Raj et al., 2010). One might speculate that TORC2-SGK-1 directly influences SKN-1 activity at several levels, from transcription to protein degradation and posttranslational modifications. However, we observed that *skn-1* mRNA levels were not affected by *rict-1* and *sgk-1*, suggesting that TORC2-SGK-1 does not simply influence *skn-1* expression. Notably, it was shown in previous studies that SKN-1 activity is regulated through phosphorylation in adult worms. SGK-1 and AKT-1/2 phosphorylated SKN-1, and repressed its nuclear accumulation and activation (Tullet et al., 2008). Alternatively the interaction could be more indirect, if both SKN-1 and TORC2 impinge on common processes. In agreement with this hypothesis, we observed that double knockout of *rict-1*(*mg451*) and *skn-1*(*tm3411*) predicted null mutants still resulted in a fraction of viable embryos. This result argues towards an alternative factor involved in mesendodermal development. TORC2 might provide a permissive environment for the specification of mesendoderm fate by SKN-1 and possibly other factors. Further work is needed to identify these factors and determine their functions in TORC2-signaling.

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Of note, previous studies indicated that the TOR kinase *let-363* and *ruvb-1*, an AAA +ATPase homolog, can suppress *pha-4* associated lethality. Mutation of *let-363/TOR* and *ruvb-1* restored pharynx development in a hypomorphic *pha-4* strain, suggesting that TOR antagonizes *pha-4* (Sheaffer et al., 2008; Updike and Mango, 2007). Whether this is primarily a TORC1 effect or also involves TORC2 is so far unknown. The transcription factor PHA-4 has an essential role in the embryonic development of the pharynx, but also acts later in life to regulate growth and longevity (Gaudet and Mango, 2002; Panowski et al., 2007). Here, we have described TORC2-mediated suppression of a more upstream component in the early EMS lineage. A speculative possibility is that there might be a common mechanism in which TORC2-mediated de-repression of a SKN-1 like activity increases *pha-4* expression, thereby allowing pharynx development.

TORC2 but not TORC1 suppresses skn-1 associated embryonic lethality

We observed that inactivation of the TORC2 core components *rict-1*, *let-363/TOR*, *lst-8*, and *sinh-1* rescued *skn-1* embryonic lethality. Of note, LST-8 is part of both complexes, TORC1 and TORC2. Yet, previous studies have demonstrated that Lst8 functions exclusively in TORC2 and that mutation of *Lst8* recapitulated the *rictor* knockout phenotype in mice, flies, and *C. elegans* (Guertin et al., 2006; Jones et al., 2009; Wang et al., 2012). Interestingly, genetic inhibition of the TOR kinase *let-363* only had a mild effect on *skn-1* associated lethality. LET-363 is part of both complexes, making it difficult to distinguish individual functions. However, our genetic analyses indicate that TORC1 appears not to be involved in control of early embryonic development. As very strong inhibition causes defects in larval developmental which might confound embryonic functions (Honjoh et al., 2009; Jia et al., 2004), we tested different genetic manipulations (RNAi and mutations) to gradually reduce TORC1 functions. We show that knockdown of the TORC1 specific genes *daf-15*/Raptor and *rheb-1* did not promote *skn-1* viability. Taken together, our data show that TORC2 but not TORC1 regulates *skn-1*-dependent developmental processes.

SGK-1 functions downstream of TORC2 to modulate skn-1 activity

Our results indicate that TORC2 and SGK-1 participate in a pathway that regulates embryonic development. In rescuing *skn-1* embryonic lethality, inactivation of *sgk-1* phenocopied inactivation of TORC2 components, and *rict-1*;*sgk-1* double mutants showed no additive effect compared to single mutants. Additionally, a constitutive active *sgk-1* mutant (Jones et al., 2009) suppressed the *rict-1* embryonic phenotype, consistent with positioning *sgk-1* downstream of *rict-1/TORC2* in a common pathway.

TORC2 is believed to control cell survival and growth by phosphorylating several AGC kinases, including AKT and SGK. Emerging data emphasize the function of SGK-1 as an important downstream effector of TORC2. TORC2 signals predominantly through SGK-1 to regulate growth, fat metabolism, and reproduction, while AKT seems only be partially involved downstream of TORC2 under certain conditions in *C. elegans* (Jones et al., 2009; Soukas et al., 2009). In yeast, activation of the SGK homolog YPK2 required phosphorylation by TORC2, and seemed to mediate most of TORC2 functions (Aronova et al., 2008; Kamada et al., 2005; Niles et al., 2012). Similarly, in mammals it has been shown that TORC2 phosphorylates and activates SGK1 (Garcia-Martinez and Alessi, 2008). The physiological significance of this mTORC2-SGK interaction however is just beginning to be revealed. Our data support the hypothesis that SGK rather than AKT is a critical output of TORC2 in regulating growth and development in *C. elegans*.

Together, our data show that TORC2-SGK-1 signaling antagonizes SKN-1 to control mesendodermal embryonic development. It is particularly surprising that TORC2 influences SKN-1 in the context of both embryonic development and aging. TORC2 is involved in

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growth and metabolism (Cybulski and Hall, 2009; Jones et al., 2009; Robida-Stubbs et al., 2012; Soukas et al., 2009), and SKN-1 functions include response to environmental stres sors, regulation of development and cellular homeostasis (Bowerman et al., 1992; Wang et al., 2010). Perhaps under certain conditions it might be advantageous to mobilize these mechanisms regulated by SKN-1.

Experimental procedures

C. elegans growth conditions

C. elegans were raised at 20 °C on standard nematode growth media plates seeded with *Escherichia coli* OP50 as described (Brenner, 1974).

Strains used in this study

The following strains were used in this study: N2 Bristol (wild-type), NL2099 *rrf-3(pk1426)*, ENH190 *rict-1(mg451)* (gift from A. Soukas & G. Ruvkun, outcrossed 4 times), KQ1366 *rict-1(ft7)*, BR4774 *sgk-1(ok538)*, ENH261 *rict-1(mg451)*;*sgk-1(ok538)*, KQ1564 *sgk-1(ft15)*, ENH337 *rict-1(mg451)*;*sgk-1(ft15)*, EU1 *skn-1(zu67)/nT1[unc-?(n754);let-?]*, ENH177 *rict-1(mg451)*;*skn-1(zu67)/nT1[unc-?(n754);let-?]*, ENH226 *skn-1(zu67)/nT1[unc-?(n754);let-?]*;*sgk-1(ok538)*, ENH256 *rict-1(ft7)*;*sgk-1(ft15)*, EU31 *skn-1(zu135)/nT1[unc-?(n754);let-?]*, ENH197 *rict-1(mg451)*;*skn-1(zu135)/nT1[unc-?(n754);let-?]*, DR2381 *let-363(h111)/dpy-5*(*e61*), DR412 *daf-15(m81)/unc-24(e138)*, RB759 *akt-1(ok525)*, VC204 *akt-2(ok393)*, ENH200 *skn-1(tm3411)/nT1[unc-?(n754);let-?]* (outcrossed 4x), ENH224 *rict-1(mg451)*;*skn-1(tm3411)/nT1[unc-?(n754);let-?]*, ENH284 *sknr-1(tm2386)* (outcrossed 4x), ENH297 *rict-1(mg451)*;*sknr-1(tm2386)*, ENH298 *skn-1(zu135)/nT1[unc-?(n754);let-?]*;*sknr-1(tm2386)*, ENH299 *rict-1(mg451)*;*skn-1(zu135)/nT1[unc-?(n754);let-?]*;*sknr-1(tm2386)*. LD1151 *sknr-1(ok1216)* (4x out-crossed), ENH269 *rict-1(mg451)*;*sknr-1(ok1216)*, ENH278 *skn-1(zu67)/nT1[qIs51]*;*sknr-1(ok1216)*, ENH279 *rict-1(mg451)*;*skn-1(zu67)/nT1[qIs51];sknr-1(ok1216)*. JR1130 *wIs84[elt-2::NLS::GFP::lacZ,rol-6D]*, MS94 *cuIs1(ceh-22::GFP;rol-6)*, MS730 *wIs93[med-1::GFP::MED-1]*. Integrated GFP reporters for *elt-2*, *ceh-22*, and *med-1* were obtained from M. Maduro. ENH271 *rict-1(mg451)*;*wIs84[elt-2::NLS::GFP::lacZ rol-6D]*, ENH272 *rict-1(mg451);cuIs1(ceh-22::GFP;rol-6)*, ENH273 *rict-1(mg451);wIs93[med-1::GFP::MED-1]* .

Double mutants *rict-1*;*skn-1* and *skn-1*;*sgk-1* were created by mating male *rict-1* or *sgk-1* homozygous with *skn-1/nT1[unc-?(n754);let-?]* heterozygous hermaphrodites that have an Unc phenotype. After transgenic males were successfully crossed twice with *skn-1/nT1* hermaphrodites F3 Unc progeny was selected for maintenance. Similarly, triple mutants *rict-1*;*skn-1*;*sknr-1* were created by mating *rict-1*;*sknr-1* males with *skn-1/nT1[unc-?(n754);let-?]*. All mutations were confirmed by PCR and sequencing.

RNA interference

RNAi by bacterial feeding was performed as described (Kamath et al., 2001). HT115 bacteria expressing the appropriate construct or pPD129.36 as empty vector control were grown in overnight cultures containing 12.5 μg/ml tetracycline and 50 μg/ml ampicillin. On the following day, cultures were diluted, grown to an OD600 of 1, and induced with 1 mM IPTG. This culture was seeded onto NGM plates containing 1 mM IPTG, ampicillin and tetracycline. For simultaneous knockdown of *skn-1* and TOR complex components RNAi cultures were mixed 1:1. The *rrf-3*(*pk1426*) RNAi supersensitive strain was used to enhance the effectiveness of targeting multiple genes.

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RNAi clones for *rict-1*, *sgk-1*, *rheb-1*, *sinh-1*, and *wdr-23* were derived from the ORF-RNAi library v1.1. *skn-1* was obtained from K. Blackwell (Tullet et al., 2008), *let-363* was previously described (Vellai et al., 2003). For *lst-8* and *daf-15* RNAi partial cDNA was cloned into pPD129.36. All clones were confirmed by sequencing.

Brood size and larval survival

Hermaphrodites were allowed to lay eggs for 4–6 h on OP50 or RNAi, and progeny were grown to the L4 stage. To assess the brood size and viable offspring, individual L4 larvae were then placed onto fresh NGM or RNAi plates. Worms were transferred every other day onto new NGM or RNAi plates until egg-laying ceased. The total number of progeny and viable offspring of each animal were counted one to three days after removal of the parental generation. All experiments were performed at 20 °C.

For experiments with *skn-1* mutants, heterozygous *skn-1* hermaphrodites bearing the *nT1* balancer and showing Unc phenotype were allowed to lay eggs. Homozygous F1 non-Unc *skn-1* L4 larvae were then used for analysis of the brood size and embryonic viability. The *zu67* and *zu135* alleles behave as strict maternal-effect lethal mutations, all progeny produced by self-fertilization of homozygous hermaphodites arrested during embryogenesis (Bowerman et al., 1992).

Microscopy and immunofluorescence

For analysis of mesodermal and endodermal development *ceh-22::GFP* (Okkema and Fire, 1994), *med-1::GFP* (Maduro et al., 2001), and *elt-2::GFP* (Fukushige et al., 1998) transgenic worms were fed with *skn-1*(RNAi) or *control*(RNAi) starting from L1. Embryos were collected from adult hermaphrodites, transferred to an agar pad with M9 buffer and allowed to develop for an interval of time equivalent to normal embryogenesis at 20 °C.

Light microscopy was performed using a Zeiss Axioplan 2-microscope equipped with Nomarski *differential interference contrast* (DIC), an AxioCam-camera and the AxioVision-Software Rel.4.8. GFP was detected using appropriate EGFP-filter sets (480/20-nm excitation, 510/20-nm emission). Images were processed with Adobe Photoshop Version 12.0. and figures compiled with Adobe Illustrator CS5.

RNA isolation and quantitative PCR

For RNA preparation 300–500 young adult worms were picked to a clean NGM plate to minimize bacterial contamination and washed several times with M9 buffer. Total RNA extraction was performed with Trizol (Sigma) followed by purification with RNA Clean & Concentrator (Zymo Research) and DNAse treatment (Quiagen). The total concentration and purity of RNA was determined by absorbance at 260/280 nm (NanoDrop spectrophotometer). cDNA synthesis was performed with 250 ng RNA using Superscript III Kit (Invitrogen).

Quantitative PCR reactions were performed with qPCR Master-Mix Plus w/o UNG for SYBR® Assay ROX (Eurogentec) and the following primers: *skn-1*_for gaagttgtaccaaacgatgtgttcc and *skn-1*_rev tgctgttgacgtcctgaagatcca. *Y45F10D.4*_for gtcgcttcaatcagttcagc and *Y45F10D.4*_rev gttcttgtcaagtgatccgaca. *cdc-42*_for ctgctggacaggaagattacg and *cdc-42*_rev ctcggacattctcgaatgaag. Experiments were performed in duplicates and carried out using Roche Light-Cycler 480. The threshold cycle (Ct) values of the target genes were analyzed and normalized to the reference genes *Y45F10D.4* and *cdc-42* (Hoogewijs et al., 2008). Relative *skn-1* expression of mutant strains was compared to wild-type using the $2^{-\Delta\Delta Ct}$ method.

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Statistical methods

Statistical analyses were carried out with SigmaStat 3.5. Comparing more than two groups, statistical analysis was performed with non-parametric Kruskal–Wallis one-way analysis of variance. Non-parametric Mann–Whitney (also called Wilcoxon rank-sum) test was used for pair-wise comparisons.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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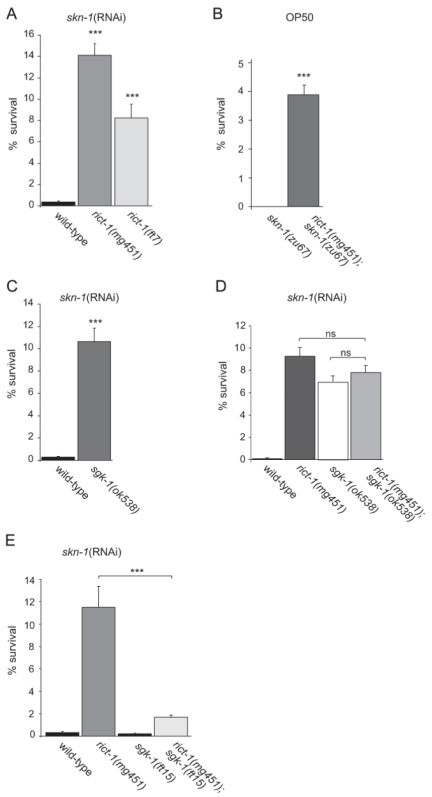

Fig. 1.

Loss of *rict-1* rescues lethality in *skn-1* deficient embryos and acts in a pathway with *sgk-1*. (A) *skn-1* embryonic lethality is suppressed by mutation of *rict-1*. RNAi feeding was used to inactivate *skn-1* function in wild-type and *rict-1* mutants starting from L1. Their total progeny and the portion that survived and progressed past the first larval stage (L1) was scored. The progeny of $n>19$ adult hermaphrodites for each condition was analyzed. Error bars indicate SEM. ***$p<0.0001$ versus wild-type on *skn-1* (RNAi). See also Table 1. (B) Quantification of the fraction of survivors of *rict-1*;*skn-1* double mutants. *rict-1(mg451)*;*skn-1(zu67)* and *skn-1(zu67)* mutants were grown on OP50 and the fraction of embryos that survived beyond L1 was counted. $n>39$ for each strain. Error bars, SEM. ***$p<0.0001$ versus *skn-1(zu67)*. See also Table 2. (C) *sgk-1* mutants suppress lethality of *skn-1* deficient embryos. RNAi feeding starting from L1 in wild-type and *sgk-1(ok538)* mutants. Quantification of larval survival per total progeny. $n>45$ for each strain. Error bars indicate SEM. ***$p<0.0001$ compared to *skn-1*(RNAi). See also Table 1. (D) Quantification of larval survival of wild-type, *rict-1(mg451)*, *sgk-1(ok538)*, and *rict-1(mg451)*;*sgk-1(ok538)* fed with *skn-1*(RNAi). $n>18$ for each strain. Error bars indicate SEM. ns, not significant. See also Table 1. (E) *sgk-1(ft15)* gain-of-function mutants suppress the viability of *rict-1*;*skn-1*(RNAi) embryos. Quantification of the fraction of survivors of wild-type, *rict-1(mg451)*, *sgk-1(ft15)*, and *rict-1(mg451)* ;*sgk-1(ft15)* fed with *skn-1*(RNAi). $n>20$ for each strain. Error bars indicate SEM. ***$p<0.0001$. See also Table 1.

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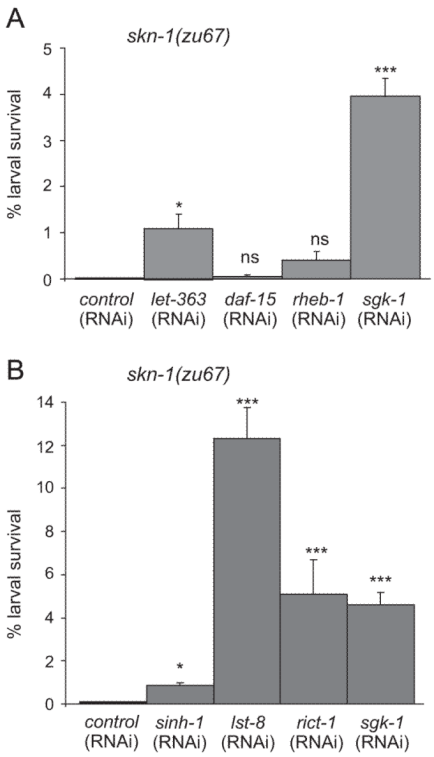

Fig. 2.

TORC2 but not TORC1 can suppress lethality of *skn-1* deficient embryos. (A) Inhibition of TORC1 components cannot suppress *skn-1*(−) embryonic lethality. *skn-1(zu67)* mutants were fed with *daf-15/CeRaptor*(RNAi), *rheb-1*(RNAi), *let-363/CeTOR*(RNAi), and *sgk-1*(RNAi). Quantification of the fraction of survivors. $n>20$ per each condition. Error bars indicate SEM. *$p<0.05$, ***$p<0.001$. ns, not significant. See also Table 3. (B) Inhibition of TORC2 promotes *skn-1*(−) embryonic viability. Quantification of survival of *skn-1(zu67)* mutant embryo fed with *rict-1*(RNAi), *sinh-1*(RNAi), *lst-8*(RNAi), and *sgk-1*(RNAi). $n>20$ per each condition. Error bars indicate SEM. *$p<0.05$, ***$p<0.001$. See also Table 3.

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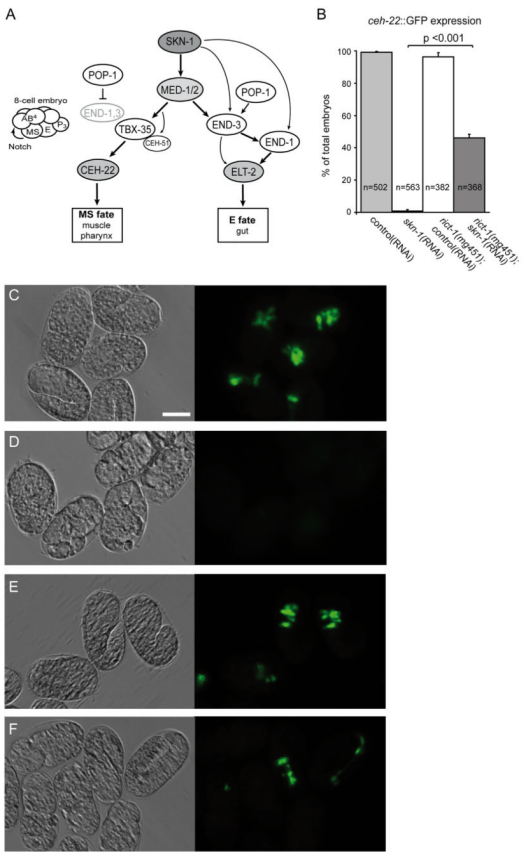

Fig. 3.

Rescue of defective mesodermal development in *skn-1* embryos by inactivation of *rict-1*. (A) The *C. elegans* gene network for specification of mesoderm and endoderm through SKN-1 and the Wnt-effector POP-1, modified from Maduro (2009). (B) Embryos lacking *rict-1* and *skn-1* function make MS-type pharynx. Wild-type and *rict-1(mg451)* mutants bearing the integrated *cuIs1[ceh-22::GFP]* marker were fed with control(RNAi) or *skn-1*(RNAi) as indicated. Bars show percentage of terminally arrested embryos positive for *ceh-22*::GFP signal. Error bars, SEM. *n*, number of embryos analyzed. Mann–Whitney rank sum test. ***$p<0.001$. (C–F) Paired DIC (left) and *ceh-22*::GFP-fluorescence images (right) of representative embryos. Scale bar represents 20 μm. (C) In wild-type embryos the *ceh-22*::GFP marker shows broad induction of mesodermal tissue. (D) *skn-1*(RNAi) embryos arrest development and completely lack *ceh-22*::GFP expression. (E) In *rict-1(mg451)* mutants *ceh-22*::GFP expression is similar to wild-type. (F) *rict-1(mg451)*;*skn-1*(RNAi) embryos show pharynx specification evident by *ceh-22*::GFP expression.

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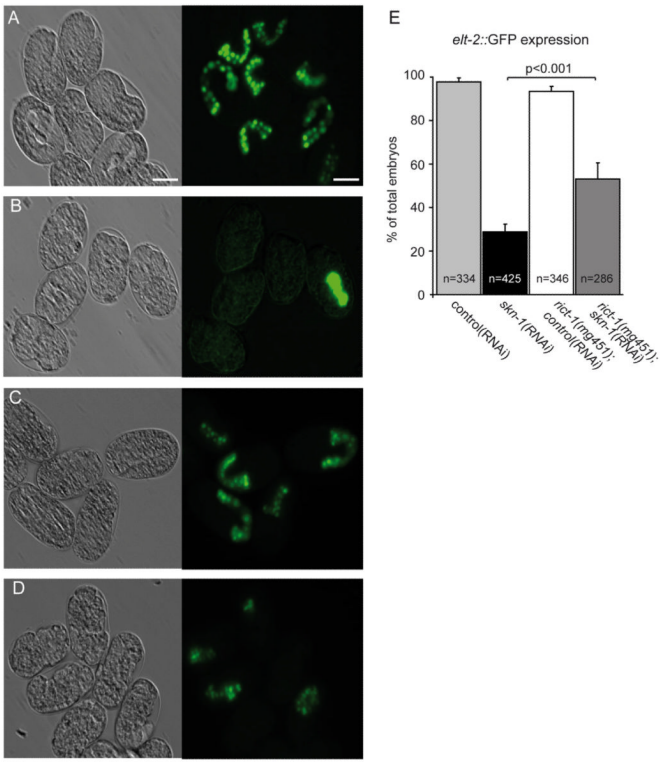

Fig. 4.

Inactivation of *rict-1* promotes endoderm development in *skn-1* deficient embryos. (A–E) Wild-type and *rict-1(mg451)* mutants carrying the integrated marker *wIs84[elt-2::NLS::GFP]* for intestinal differentiation were fed with control(RNAi) or *skn-1*(RNAi) as indicated. Paired DIC (left panel) and fluorescence images (right panel) of embryos expressing *elt-2*::GFP. Scale bar represents 20 μm. (A) Expression of the *elt-2*::GFP in the developing gut of wild-type embryos. (B) A large fraction of *skn-1*(RNAi) embryos fails to express *elt-2*::GFP and lacks endoderm. (C) In *rict-1(mg451)* mutants *elt-2*::GFP expression does not differ from wild-type. (D) Inactivation of *rict-1(mg451)* in *skn-1*(RNAi) embryos induces *elt-2*::GFP expression. (E) Quantification of *elt-2*::GFP positive embryos from A-D. Significantly more *rict-1* mutants fed with *skn-1*(RNAi) develop *elt-2*::GFP positive gut cells compared to *skn-1*(RNAi). Mann–Whitney rank sum test, $p<0.001$. Error bars, SEM. *n*, number of embryos analyzed.

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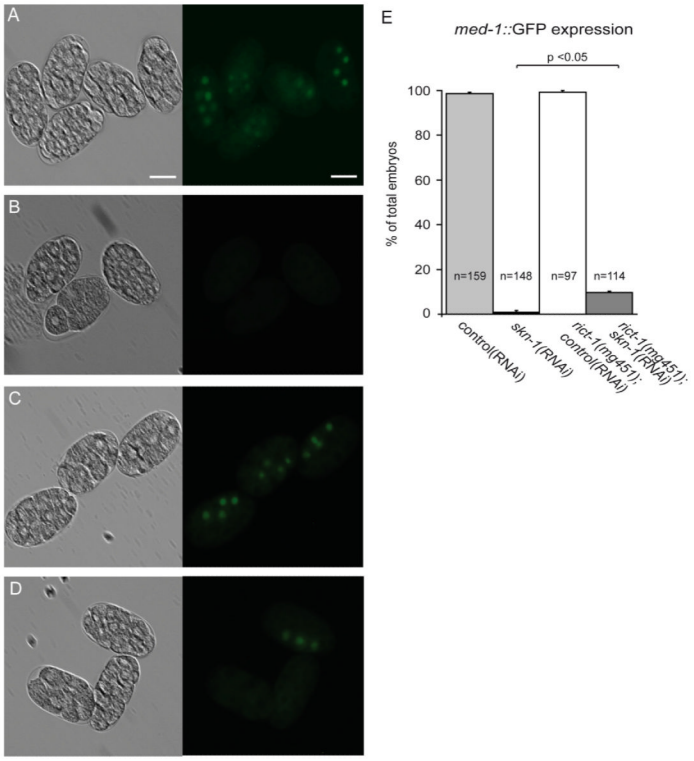

Fig. 5.

Loss of *rict-1* activates *med-1* in embryos lacking *skn-1*. (A–E) Wild-type and *rict-1(mg451)* mutants carrying the integrated marker *wIs93[med-1::GFP]* were fed with control(RNAi) or *skn-1*(RNAi) as indicated. Paired DIC (left) and fluorescence images (right). Scale bar represents 20 μm. (A) *med-1*::GFP expression in wild-type embryos. (B) *skn-1*(RNAi) embryos arrest development and completely lack *med-1*::GFP expression. (C) In *rict-1(mg451)* mutants *med-1*::GFP expression is similar to wild-type. (D) Embryos grown on *rict-1(mg451)*;*skn-1*(RNAi) show an increased *med-1*::GFP expression. (E) Quantification of *med-1::GFP* positive embryos from A–D. Substantially more *rict-1* mutants fed with *skn-1*(RNAi) display *med-1*::GFP positive cells compared to *skn-1*(RNAi) embryos. Mann–Whitney rank sum test. $p<0.05$. Error bars, SEM. *n*, number of embryos scored.

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Table 1

Loss of *rict-1* rescues lethality in *skn-1* deficient embryos and acts in a pathway with *sgk-1*. Corresponds to Fig. 1A, C–E. RNAi feeding was used to inactivate *skn-1* function in the indicated wild-type and mutant strains starting from L1. Their total progeny and the portion that survived and progressed past the first larval stage (L1) was scored. N represents the number of adult hermaphrodites used for analysis of their progeny.

Strain	RNAi	*n* (Adult worms)	Mean number of Eggs±SEM	% Survival±SEM	*P* value versus control	Figure
N2	skn-1	20	237±8	0.4±0.1		1A
rict-1(mg451)	skn-1	19	120±3	14.1±1.1	<0.0001[a]	1A
rict-1(ft7)	skn-1	19	90±3	8.2±1.3	<0.0001[a]	1A
N2	skn-1	45	256±4	0.3±0.1		1C
sgk-1(ok538)	skn-1	48	122±3	10.7±1.2	<0.0001[a]	1C
N2	skn-1	20	245±6	0.1		1D
rict-1(mg451)	skn-1	20	141±2	9.3±0.8	<0.0001[a]	1D
sgk-1(ok538)	skn-1	18	139±4	6.8±0.5	<0.0001[a]	1D
rict-1(mg451);sgk-1(ok538)	skn-1	19	149±3	7.8±0.6	ns[b], ns[c], <0.0001[a]	1D
N2	skn-1	20	238±12	0.3±0.1		1E
rict-1(mg451)	skn-1	24	109±6	11.5±1.9	<0.0001[a]	1E
sgk-1(ft15)	skn-1	20	203±18	0.2±0.1	ns[a]	1E
rict-1(mg451);sgk-1(ft15)	skn-1	24	154±5	1.7±0.2	<0.0001[b]	1E
sgk-1(ft15)	control	5	243±11	100		
rict-1(mg451);sgk-1(ft15)	control	5	122±10	100		
N2	skn-1	10	275±6	0.6±0.1		
akt-1(ok525)	skn-1	10	198±8	0.6±0.2	ns[a]	
akt-2(ok393)	skn-1	10	210±14	0.5±0.1	ns[a]	
rict-1(mg451)	skn-1	10	113±6	8.8±0.8	<0.0001[a]	

ns, not significant. SEM, standard error of the mean.

[a] *p* value was calculated versus N2 *skn-1*(RNAi).

[b] *p* value was calculated versus *rict-1(mg451), skn-1*(RNAi).

[c] *p* value was calculated versus *sgk-1*(ok538):*skn-1*(RNAi).

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Table 2

Quantification of the fraction of survivors of *rict-1*;*skn-1* and *skn-1*;*sgk-1* double mutants. Indicated strains were grown on OP50 and the fraction of embryos that survived beyond L1 was counted.

Strain	*n* (Adult worms)	Mean number of Eggs±SEM	% Survival ±SEM	*P* value versus control	Figure
N2	5	272±12	100		
rict-1(mg451)	5	116±4	100		
skn-1(zu67)	40	232±10	0		1B
rict-1(mg451);skn-1(zu67)	39	119±4	3.9±0.3	<0.0001[a]	1B
skn-1(zu135)	24	272±13	0		
rict-1(mg451);skn-1(zu135)	19	155±13	2.4±0.7	<0.0001[b]	
rict-1(ft7)	5	91±2	100		
skn-1(zu67)	10	273±6	0		
rict-1(ft7);skn-1(zu67)	13	154±7	3.0±0.5	<0.0001[a]	
sgk-1(ok538)	5	139±4	100		
skn-1(zu67)	23	259±9	0		
skn-1(zu67);sgk-1(ok538)	24	145±6	1.8±0.2	<0.0001[a]	

[a] *p* value was calculated versus *skn-1*(*zu67*).

[b] *p* value was calculated versus *skn-1*(*zu135*).

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Table 3

TORC2 but not TORC1 can suppress lethality of *skn-1* deficient embryos. Indicated strains were fed with RNAi and the fraction of embryos that survived beyond L1 was counted.

Strain	**RNAi**	***n* (Adult worms)**	**Mean number of Eggs ±SEM**	**% Survival±SEM**	***P* value versus control**	**Figure**
skn-1(zu67)	control	34	211±10	0		2A
skn-1(zu67)	let-363	42	65±5	1.1±0.3	<0.05[a]	2A
skn-1(zu67)	daf-15	33	132±9	0.05±0.04	ns[a]	2A
skn-1(zu67)	rheb-1	23	118±11	0.4±0.2	ns[a]	2A
skn-1(zu67)	sgk-1	35	100±6	3.9±0.5	<0.001[a]	2A
skn-1(zu67)	control	38	203±9	0		2B
skn-1(zu67)	sinh-1	39	152±6	0.8±0.1	<0.05[a]	2B
skn-1(zu67)	lst-8	35	58±4	12.3±1.5	<0.001[a]	2B
skn-1(zu67)	rict-1	20	89±6	5.2±1.6	<0.001[a]	2B
skn-1(zu67)	sgk-1	40	98±5	4.6±0.6	<0.001[a]	2B
N2	skn-1	29	227±8	0.2±0.1		Suppl.3
let-363(h111)/+	skn-1	30	166±12	1.9±0.3	<0.05[c]	Suppl.3
daf-15(m81)/+	skn-1	30	207±8	0.7±0.2	ns[c]	Suppl.3
rict-1(mg451)	skn-1	29	87±3	10.5±1.0	<0.001[c]	Suppl.3
Strain	Combined RNAi	*n* (Adult worms)	Mean number of Eggs ±SEM	% Survival±SEM	*P* value versus control	
rrf-3(pk1426)	control;skn-1	30	68±3	0.2±0.1		
rrf-3(pk1426)	rheb-1;skn-1	21	53±4	0.1±0.1	ns[b]	
rrf-3(pk1426)	rict-1;skn-1	29	65±5	3.2±0.9	<0.001[b]	
rrf-3(pk1426)	sgk-1;skn-1	29	53±4	9.0±1.1	<0.001[b]	
rrf-3(pk1426)	control;skn-1	39	72±3	0.4±0.2		
rrf-3(pk1426)	daf-15;skn-1	36	48±3	0.5±0.2	ns[b]	
rrf-3(pk1426)	rict-1;skn-1	39	71±5	2.7±0.7	<0.001[b]	
rrf-3(pk1426)	sgk-1;skn-1	39	53±4	14.0±2.3	<0.001[b]	
rrf-3(pk1426)	control;skn-1	28	70±3	0.3±0.1		
rrf-3(pk1426)	sinh-1;skn-1	29	68±3	4.1±0.8	<0.001[b]	
rrf-3(pk1426)	rict-1;skn-1	28	65±4	3.7±0.8	<0.001[b]	
rrf-3(pk1426)	sgk-1;skn-1	19	68±3	8.5±1.4	<0.001[b]	

ns, not significant.

[a] *p* value was calculated versus *skn-1(zu67)* on control(RNAi).

[b] *p* values wase calculated versus *rrf-3(pk1426)* on control;*skn-1*(RNAi).

[c] *p* values was calculated versus N2 on *skn-1*(RNAi).

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Table 4

sknr-1 cannot compensate for loss of *skn-1* function. Strains were fed with RNAi or OP50 as indicated and the fraction of embryos that survived beyond L1 was counted.

Strain		***n*** **(Adult worms)**	**Mean number of Eggs±SEM**	**% Survival±SEM**	**P value versus control**
rict-1(mg451);skn-1(zu135);sknr-1(tm2386)	OP50	14	142±4	2.0±0.3	ns[a], <0.001[b]
rict-1(mg451);skn-1(zu135)	OP50	10	141±9	2.2±0.1	<0.001[b]
skn-1(zu135);sknr-1(tm2386)	OP50	10	269±5	0	
skn-1(zu135)	OP50	10	304±14	0	
sknr-1(tm2386)	OP50	8	233±13	100	
rict-1(mg451);skn-1(zu67);sknr-1(ok1216)	OP50	26	103±12	4.2±0.5	ns[c], <0.001[d]
rict-1(mg451);skn-1(zu67)	OP50	26	95±19	4.5±0.5	<0.001[d]
skn-1(zu67);sknr-1(ok1216)	OP50	26	206±14	0	
skn-1(zu67)	OP50	27	211±14	0	
sknr-1(ok1216)	OP50	5	254±16	100	
skn-1(tm3411)	HT115	5	200±14	0	
rict-1(mg451);skn-1(tm3411)	HT115	10	139±6	2.4±0.1	<0.001[g]
Strain	RNAi	*n* (Adult worms)	Mean number of Eggs±SEM	% Survival±SEM	*P* value versus control
N2	skn-1	20	240±9	0.5±0.1	
rict-1(mg451)	skn-1	19	113±4	14.5±1.3	<0.001[e]
rict-1(mg451);skn-1(zu67)	control	27	133±6	5.2±0.4	<0.001[e]
rict-1(mg451);skn-1(zu67)	skn-1	27	112±6	2.8±0.2	<0.01[f], <0.01[e]
skn-1(zu67)	control	15	218±6	0	
skn-1(zu67)	wdr-23	20	1±3±8	0.1	ns[h]
rict-1(mg451);skn-1(zu67)	control	20	109±5	4.6±0.5	<0.001[h]
rict-1(mg451);skn-1(zu67)	wdr-23	20	106±4	4.0±0.3	ns[i], <0.001[h]

ns, not significant.

[a] *p* value was calculated versus *rict-1(mg451);skn-1(zu135)*.

[b] *p* value was calculated versus *skn-1(zu135)*.

[c] *p* value was calculated versus *rict-1(mg451);skn-1(zu67)*.

[d] *p* value was calculated versus *skn-1(zu67)*.

[e] *p* value was calculated versus N2 on *skn-1*(RNAi).

[f] *p* value was calculated versus *rict-1(mg451);skn-1(zu67)*;control(RNAi).

[g] *p* value was calculated versus *skn-1(tm3411)*.

[h] *p* value was calculated versus *skn-1(zu67)*;control(RNAi).

[i] *p* value was calculated versus *rict-1(mg451);skn-1(zu67)*;control(RNAi).