Mol. Cells 2016; 39(11): 827-833
http://dx.doi.org/10.14348/molcells.2016.0222

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Allele-Specific Phenotype Suggests a Possible Stimulatory Activity of RCAN-1 on Calcineurin in *Caenorhabditis elegans*

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Regulator of calcineurin 1 (RCAN1) binds to calcineurin through the PxIxIT motif, which is evolutionarily conserved. SP repeat phosphorylation in RCAN1 is required for its complete function. The specific interaction between RCAN1 and calcineurin is critical for calcium/calmodulin-dependent regulation of calcineurin serine/threonine phosphatase activity. In this study, we investigated two available deletion *rcan-1* mutants in *Caenorhabditis elegans*, which proceed differently for transcription and translation. We found that *rcan-1* may be required for calcineurin activity and possess calcineurin-independent function in body growth and egg-laying behavior. In the genetic background of enhanced calcineurin activity, the *rcan-1* mutant expressing a truncated RCAN-1 which retains the calcineurin-binding PxIxIT motif but misses SP repeats stimulated growth, while *rcan-1* lack mutant resulted in hyperactive egg-laying suppression. These data suggest *rcan-1* has unknown functions independent of calcineurin, and may be a stimulatory calcineurin regulator under certain circumstances.

INTRODUCTION

Regulator of CAlcineuriN 1 (RCAN1) is a highly conserved protein, first identified in fungi and yeasts as an inhibitory regulator to calcineurin, a calcium/calmodulin-dependent phosphatase (Gorlach et al., 2000; Kingsbury and Cunningham, 2000). RCAN1 is highly expressed in the striated muscle, the nervous system and induced, during pathological conditions such as neurodegeneration (Crawford et al., 1997; Fuentes et al., 2000; Wiese et al., 1995; Yang et al., 2000). RCAN1 suppresses dephosphorylation of a multi-functional transcription factor NFAT, which is a well-known substrate for calcineurin, and enhanced calcineurin activity induces RCAN1 expression via negative feedback (Fuentes et al., 2000; Yang et al., 2000). Therefore, RCAN1 overexpression due to trisomy 21 in Down syndrome plays a critical role in the pathology, because of RCAN1's inhibitory interaction with calcineurin (Ermak et al., 2001; Fuentes et al., 1995; 1997). However, there is evidence that RCAN1 is also required for optimal calcineurin activity. Low calcineurin activity was observed in RCAN1 null mutant yeast, accompanied by phenotypes similar to RCAN1-overexpressing cells (Kingsbury and Cunningham, 2000). In addition, relatively low RCAN1 expression in physiological conditions activates the calcineurin pathway, when RCAN1 is functionally phosphorylated at highly conserved serine sites (Hilioti et al., 2004; Kingsbury and Cunningham, 2000; Li et al., 2015). Therefore, RCAN1's regulatory modes on calcineurin is complicated, depending on the cellular and developmental contexts of organisms, and the biochemical and molecular properties of RCAN1.

Down syndrome model transgenic mice overexpressing RCAN1 exhibit a variety of phenotypic features that mimic Down syndrome such as cranial structure malformation, learning and memory deficits, disorganized neuronal differentiation and tumorigenesis suppression (Dierssen et al., 2011; Kurabayashi and Sanada, 2013; Martin et al., 2012; Reynolds et al., 2010). Interestingly, RCAN1 knock-out mice also show phenotypes similar to transgenic mice, such as impaired spatial learning and memory, and abnormal long-term potentiation (Hoeffer et al., 2007). In addition, both RCAN1 knock-out mice and RCAN1-1S TG mice displayed reduced exocytosis levels (Keating et al., 2008). Neuronal differentiation is also defective in transgenic flies overexpressing RCAN1 and loss-of-function mutant flies, presumably due to disturbed axonal extension associated with actin dynamics dysregulation (Chang and Min, 2009; Chang et al., 2003; Wang et al., 2012; 2016). In *C. elegans*, RCAN-1 overexpression resulted in calcineurin loss-of-function-like phenotypes, such as small body size, cuticle defects, small brood size, slow growth and resistance to serotonin-mediated egg-laying, while RCAN-1 deletion mutants showed cryophilic thermotaxis behavior, which is similar to calcineurin gain-of-function mutants (Lee et al., 2003; Li et al., 2015). Therefore, finely regulated RCAN-1 expression level is critical for normal organism physiology, in which the conserved calcineurin/RCAN1 pathways play a pivotal role in various biological processes.

RCAN1 binds to calcineurin through specified binding motifs. The PxIxIT motif is required for binding to calcineurin, which is also present in many calcineurin substrates such as a transcript-

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Received 13 September, 2016; revised 27 October, 2016; accepted 31 October, 2016; published online 21 November, 2016

Keywords: calcineurin, *C. elegans*, RCAN

eISSN: 0219-1032

tion factor NFAT (nuclear factor of activated T-cells) (Aramburu et al., 1998; Li et al., 2015; Mehta et al., 2009). RCAN1 also harbors SP repeats containing conserved serines whose phosphorylation is required for calcineurin stimulatory regulation in yeasts (Hilioti et al., 2004; Kingsbury and Cunningham, 2000). We previously reported the highly conserved PxIxIT motif, not the less conserved PKIIQT motif, is critical for full RCAN1 inhibitory regulation on *TAX-6*/calcineurin in *C.elegans*. Interestingly, *C.elegans* RCAN1 SP repeat phosphorylation is critical in RCAN1 *TAX-6*/calcineurin inhibitory regulation.

In this study, we investigated two available *C.elegans rcan-1* deletion mutants, *rcan-1(tm1925)* and *rcan-1(tm2021)*, in which RCAN was not detected by western blot analysis (Li et al., 2015). Both mutants showed normal brood size, serotonin-stimulated egg-laying, and male tail structure. However, *rcan-1(tm1925)* mutants were smaller than wild type animals, while *rcan-1(tm2021)* mutants were comparable. Also, the deletion mutation in *rcan-1(tm1925)* made *tax-6(lf)* animals even smaller, whereas that in *rcan-1(tm2021)* made *tax-6(gf)* bigger. Also, hyperactive egg-laying in *tax-6(gf)* was suppressed in both *rcan-1(tm1925)* and *rcan-1(tm2021)* mutant background. We present evidence that *rcan-1(tm2021)* may produce truncated proteins containing the PxIxIT motif, but are missing SP repeats at low expression levels, whereas *rcan-1(tm1925)* is a true null mutant. This study suggests that *rcan-1* might be either an inhibitory or a stimulatory calcineurin regulator in different genetic and biological contexts in *C. elegans*.

MATERIALS AND METHODS

Strains

Wild type N2 and *tax-(ok2065)* were obtained from Caenorhabditis Genetics Center (CGC) at the University of Minnesota. *rcan-1* mutants, *rcan-1(tm1925)* and *rcan-1(tm2021)* were obtained from National Bioresource Project Japan. *tax-6(jh107)* was isolated by standard TMP/UV mutagenesis (Park et al., 2001a).

Transgenic lines

pPD95.77 and pPD49.26 were generous gifts from Andy Fire. To make a RCAN-1 translational expression construct, a *rcan-1* genomic sequence, as well as approximately 3 kb of 5′ upstream sequence was cloned into pPD95.77. Primers were previously described (Li et al., 2015).

Immunostaining

Immunostaining against RCAN-1 was performed as previously described (Park et al., 2001b).

Serotonin stimulated egg-laying assay

Serotonin stimulated egg-laying was conducted as previously described with some modification (Trent et al., 1983). Worms were placed in 96-well plates containing M9 buffer either with or without 12.5 mM serotonin. After 90 min, the number of eggs laid by each worm was counted.

Body size

Worms were synchronized at L4, and then adult body sizes were measured using a Zeiss microscope and Axio Image software.

RESULTS

rcan-1 expression pattern and mutant information

rcan-1 was expressed in various tissues including male tail and hypodermal seam cells, which also highly expressed *tax-6*/calcineurin (Bandyopadhyay et al., 2002; Lee et al., 2003). To further explore in which organs *rcan-1* may physiologically function, we expressed RCAN-1 GFP fusion protein driven by its own promoter in worms. RCAN-1::GFP is expressed in various

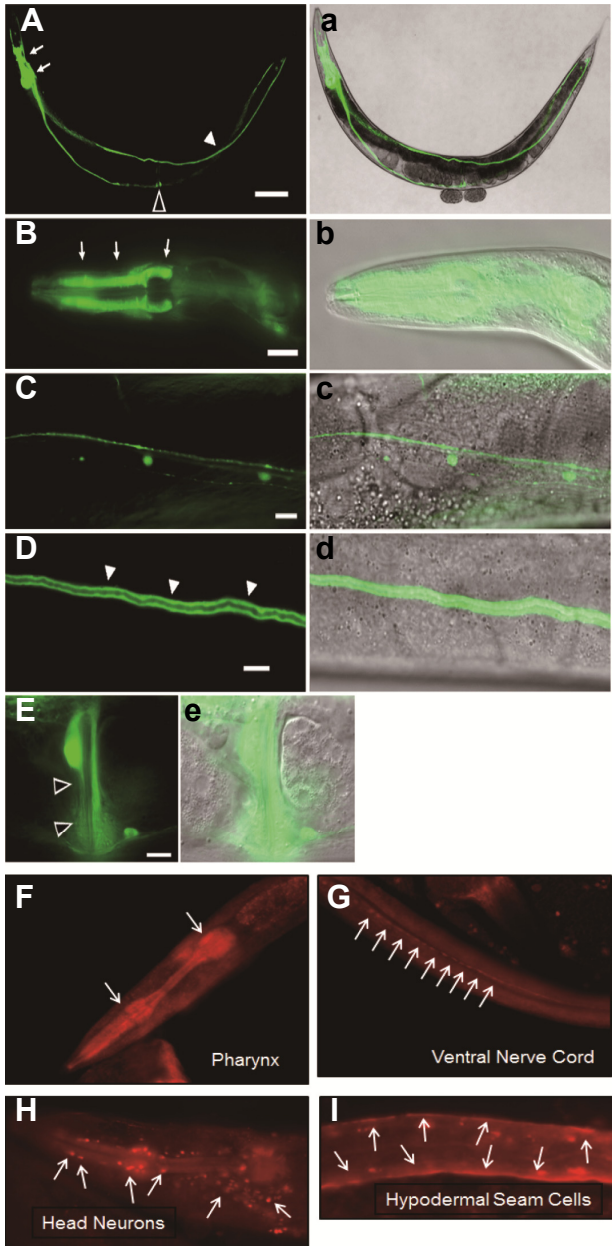

Fig. 1. RCAN-1 expression in *C. elegans*. Fluorescence images of worms expressing RCAN-1::GFP are shown in (A-E), along with matching overlapped DIC images (a, b, c, d, and e), and immunostaining to RCAN-1 using polyclonal anti-RCAN-1 antibodies are also presented (F) to (I). RCAN-1 is expressed in various tissues, including pharyngeal muscle and head neurons (B, b, and F; arrows), ventral nerve cords (C, c, and G), excretory canal cells (D and d; arrow heads in A), vulva (E and e; an open arrow head in A), and hypodermal seam cells (I). Size bar; 100 μm in A, and 10 μm in (B-E).

head neurons, pharyngeal muscle, ventral nerve cord, and hypodermal seam cells where immunostaining for RCAN-1 was also prominent (Figs. 1A-1C, and 1F-1I). GFP fluorescence was also detected in an excretory canal cell and vulva (Figs. 1D and 1E).

rcan-1 regulates a body size and serotonin-stimulated egg-laying of *C. elegans*

Worms overexpressing RCAN-1 exhibited pleiotrophic phenotypes similar to *tax-6* loss-of-function mutants, suggesting RCAN-1 is inhibitory to *tax-6*/calcineurin (Lee et al., 2003). To further investigate *rcan-1* functions, we examined two available *rcan-1* deletion mutant alleles, *rcan-1(tm1925)* and *rcan-1(tm2021)*. These mutants have a brood size comparable to wild type animals, and a male tail with normal morphology (Supplementary Fig. 1)(Li et al., 2015). Because RCAN-1 is highly expressed in seam cells (Fig. 1I), and *tax-6* loss-of-function mutants are small (Kuhara et al., 2002), we measured body size for those two *rcan-1* alleles, asking the question whether *rcan-1* played a role in growth. *tm1925* animals were slightly smaller than wild types, while *tm2021* worms exhibited normal body length (Fig.2). We further investigated *tax-6* mutant body size and various *rcan-1;tax-6* double mutants. Both *tax-6(ok2065)* loss-of-function (lf) and *tax-6(jh107)* gain-of-function (gf) mutant worms were significantly smaller than wild types. Interestingly, *tm1925* allele did not affect *tax-6(jh107)gf*, but shrank *tax-6(ok2065)lf smaller*. However, *tm2021* allele made *tax-6(jh107)gf* bigger, but did not affect *tax-6(ok2065)lf*. In addition, those body size regulation effects were not observed in *cnb-1(jh103)* in which intact *tax-6* was present, but no calcineurin activity. To further explore differential phenotype modulation by these two alleles, we also investigated egg-laying stimulation by serotonin which is dependent on *tax-6* activity. Egg-laying activity in both *tm1925* and *tm2021* mutant worms were slightly stimulated by serotonin, though the average number of eggs laid per worm was comparable to wild types (Figs. 3A and 3D). Both alleles suppressed enhanced egg-laying of *tax-6(jh107)gf*, but only *tm1925* reversed *tax-6(ok2065)lf* reduced egg-laying activity (Fig.3B and 3D). Again, differential modulation by the two different alleles was not observed in *cnb-1(jh103)ls* background, suggesting that *tax-6* abnormal activity was associated with body length modulation by *tm1925* and *tm2021* alleles (Fig. 3C and 3D).

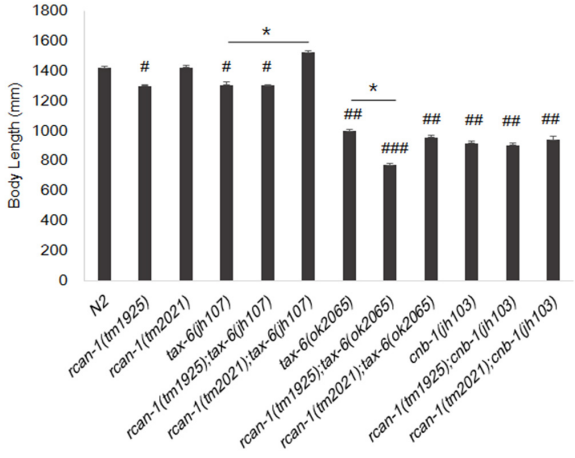

Fig. 2. *rcan-1* and *tax-6* mutant body lengths. Adult worm longitudinal size for each genetic background animal was measured and plotted. n=20, $^{*}p < 0.01$; compared to N2, $^{\#}p < 0.01$ and $^{\#\#} < 0.0001$, $^{\#\#\#} < 0.00001$; *t*-test.

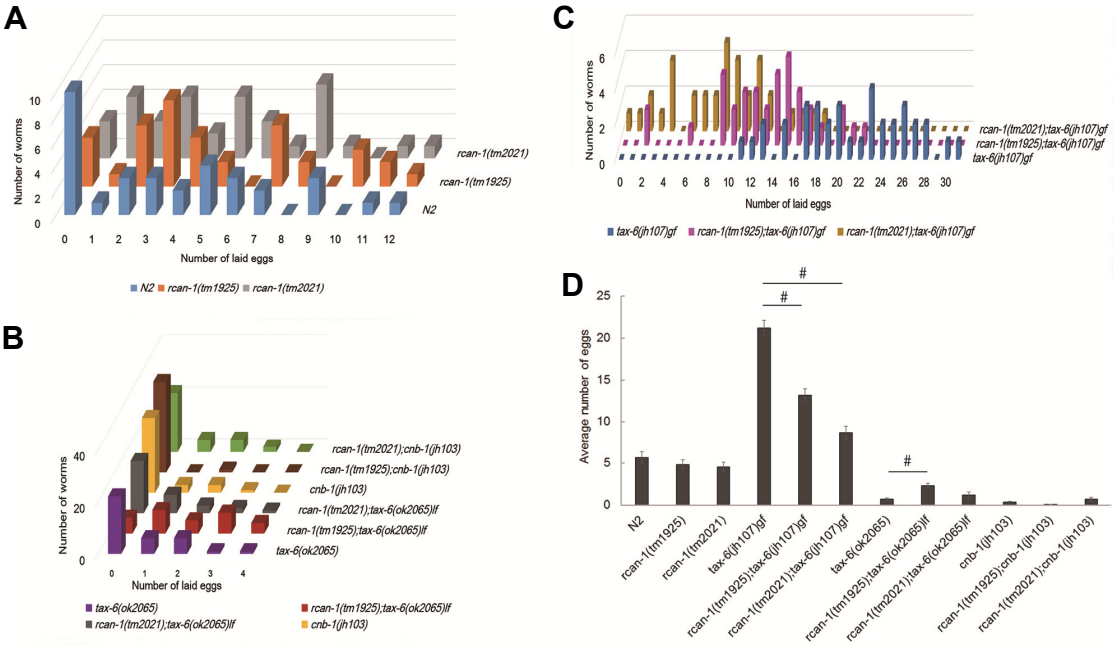

Fig. 3. *rcan-1* and *tax-6* mutant egg-laying assay. A single worm is placed and incubated for 90 minutes in each well of 96-well plates containing 12.5 mM serotonin, and laid eggs number for each worm was scored. The 36 worms for each mutant background were tested and plotted for *N2*, *rcan-1(tm1925)*, and *rcan-1(tm2021)* in (A), *tax-6(ok2065)*, *cnb-1(jh103)*, and their double mutants with *rcan-1* in (B), and *tax(jh107)* and its doubles in (C). Laid egg average number for each mutant background is plotted in (D). $^{\#}p < 0.001$, *t*-test.

tm1925 is a true null mutant whereas *tm2021* is expression-competent

The *tm1925* allele had a large deletion in the 3rd intron and part of the 4th exon, and additions of several nucleotides near the deletion site in the 4th exon, while the *tm2021* allele had a deletion in the 2nd and 3rd exons (Fig. 4A). Both alleles were once predicted to be null mutants, because no RCAN-1 protein was detected by western blot analyses (Li et al., 2015). To analyze *rcan-1* transcriptional products in each allele mutant, we amplified *rcan-1* cDNA sequences isolated from mutant worms, and sequenced the RT-PCR products (Figs. 4A-4C). Among the detected transcripts, a splicing donor site was created at the deletion site in the 3rd intron in the *tm1925* transcript A, while the 3rd intron undeleted starting region was transcribed, linking to the remaining 4th exon in the *tm1925* transcript B. Both transcripts contained a newly created stop codon (Fig. 4B). For the

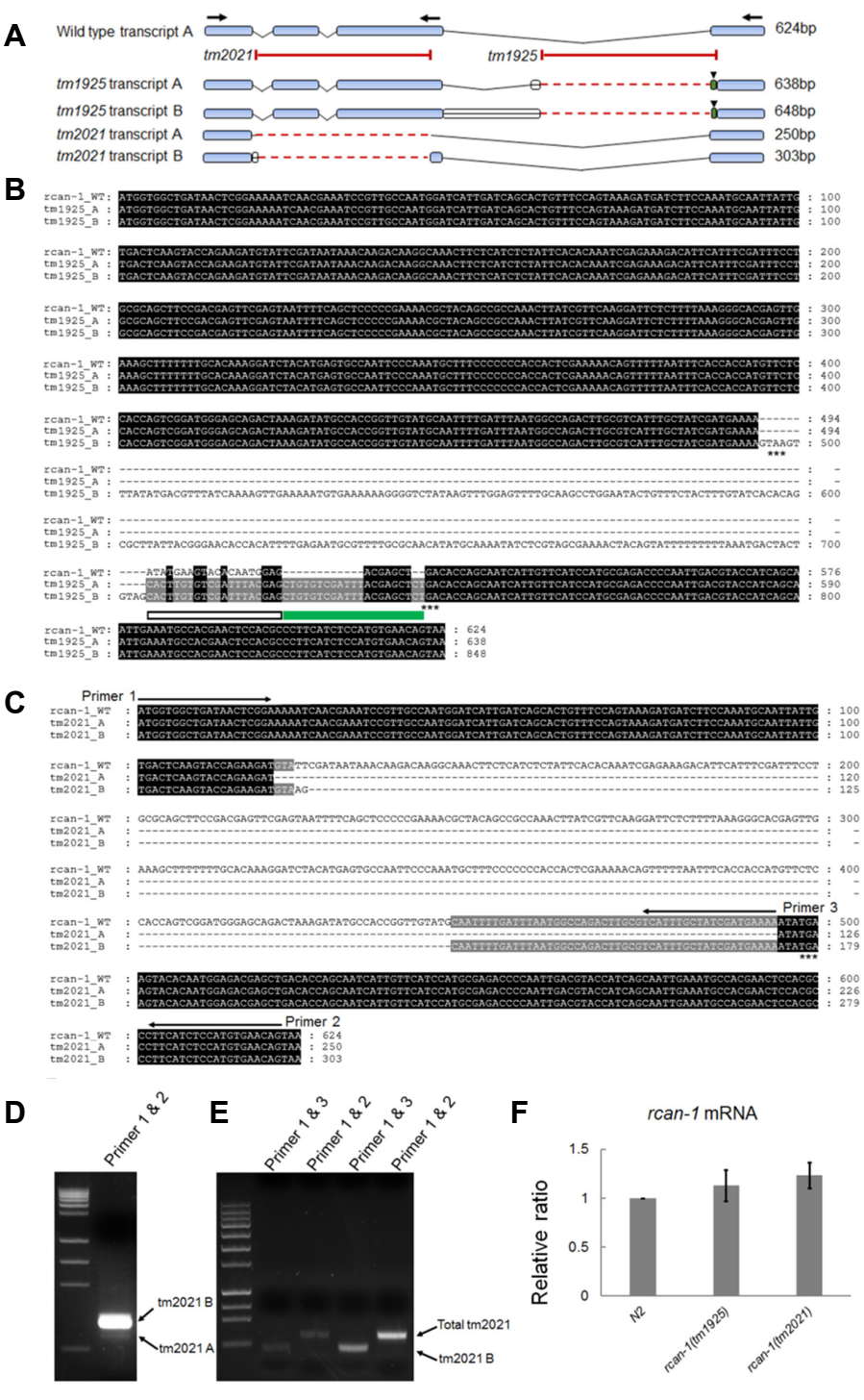

Fig. 4. *rcan-1 mutants* gene structure and transcription. (A) *rcan-1* coding region consists of 4 exons and 3 introns in between. Deletion location for each mutant is depicted as a red bar, and as red broken lines in transcripts detected from each mutant background animal. The 21bp insertion mutation sequence is indicated by arrowheads and green boxes. The 20 bp and the 234 bp intron sequences in *tm1925* transcript A and B, and the 4bp intron sequence in *tm2021* transcript B are indicated as white boxes with a black line in them. (B, C) *rcan-1* transcripts from *tm1925* and *tm 2021* mutant animals are aligned with those with from wildtypes using Clustal Omega. The stop codons generated by the mutations are indicated with asterisks: at 535-537 bp in the *tm1925* transcript A, 496-498 bp in the *tm1925* transcript B, and at 124-126 bp in the *tm2021* transcript A. The predicted *tm1925* transcript A and B translational products are approximately 20 and 18 kDa, and show calcineurin binding motif lack. *tm2021* transcript A and B were 4 and 11 kDa, respectively, and the 11 kDa one retained the calcineurin binding motif. Primer 1 and 2 set used to detect both *tm2021* transcript A and B; primer 1 and 3 set used to specifically detect *tm2021* transcript B. (D) RT-PCR showed 303 bp *tm2021* transcript B was amplified significantly more than 250 bp *tm2021* transcript A, indicating transcript B is a major transcript in *tm2021* mutant worms. (E) 28 cycle and 33 cycle semi-quantitative RT-PCR was performed using the same template amount. PCR products were run to relatively quantify the amount of *tm2021* transcript B vs. total *rcan-1* mRNA. There was no significant difference between total *tm2021* mRNA level and *tm2021* transcript B level, indicating that transcript B is dominantly produced. (F) *rcan-1* mRNA qPCR levels in WT, *tm1925* and *tm2021*. There was no significant difference between wild type, *tm1925*, and *tm2021* for *rcan-1* mRNA expression level.

tm2021 transcript, the A transcript, in which splicing between the 1st exon and the 4th exon occurred, was a major product, while the B transcript contained a piece of the 1st intron and the 3rd exon was minor (Figs. 4D-4E).

The predicted RCAN-1 translational product in *tm1925* had the PxIxIT calcineurin binding motif deleted (Figs. 4B and 5A), whereas that in *tm2021* retained it, but lost the SP repeat domain including S129 and S133 phosphorylation sites, which are critical for *tax-6* activity regulation (Li et al., 2015) (Figs. 4C and 5B). To find out whether the *rcan-1* mutant sequences were competent to produce any translational product, we cloned the mutants' genomic sequences into the GFP expression vector. We found that transgenic worms injected with the *tm2021* genomic construct exhibited reasonably detectable green fluorescence at a short exposure, whereas those with the *tm1925* showed little fluorescence even after a long exposure (Figs. 5C-5E). To detect any *rcan-1:GFP* translational products in those transgenic lines, we performed western blot analysis using polyclonal anti-RCAN-1 antibodies (Fig. 5F). 38 kDa of tm2021:GFP fusion was detected, but the predicted tm1925::GFP was not detected due to the stop codons generated by the mutation (Fig. 4B). In addition, no *tm1925* predicted products were detected, indicating that *tm1925* transcripts were probably not translated, or translational products were subject to fast degradation. Therefore, *tm1925* was likely to be a null mutant, and *tm2021* might produce a truncated protein retaining the PxIxIT motif, but missing the SP repeats domain, making expression level undetectable by typical Western blot analysis using currently available polyclonal anti-RCAN-1 antibodies.

DISCUSSION

RCAN1 has been widely shown to be an inhibitory calcineurin regulator, but several lines of studies have shown that phosphorylated RCAN1 is able to stimulate calcineurin at low expression levels (Genesca et al., 2003; Hilioti et al., 2004; Kishi et al., 2007). Also, both knock-out and overexpression lines in various models show similar malfunction in different phenotypes. This study showed that *C.elegans* rcan-1 may also play a stimulatory role in the calcineurin pathway by investigating a phenotypic variance between two deletion rcan-1 mutants.

Previously considered to be null mutants, both *rcan-1(tm1925)* and *rcan-1(tm2021)* mutants are cryophilic for *tax-6 (gf)*, suggesting that RCAN1 should be an inhibitory calcineurin regulator in thermotaxis behavior (Li et al., 2015). However, these two mutants showed differential phenotypes for body length and serotonin-stimulated egg-laying behavior (Figs. 2 and 3). The *tm1925* allele had PxIxIT calcineurin binding motif deletion, and its GFP fusion protein genomic sequence was not detectable, indicating that *tm1925* is null (Fig. 4). On the other hand, the *tm2021* was predicted to produce a truncated PxIxIT motif. When the *tm2021* genomic sequence was cloned in frame into a GFP expression vector, it produced a GFP fusion protein with expected size in transgenic worms that was detected by western blot. The *tm1925* mutant animal was shorter than wildtypes, and *rcan-1(tm1925);tax-6(ok2065)* was even shorter, indicating that RCAN1 may regulate calcineurin- independent growth. The fact that *rcan-1(tm2021);tax-6(jh107)* were longer than *tax-6(jh107)gf* also supports RCAN1's regulatory

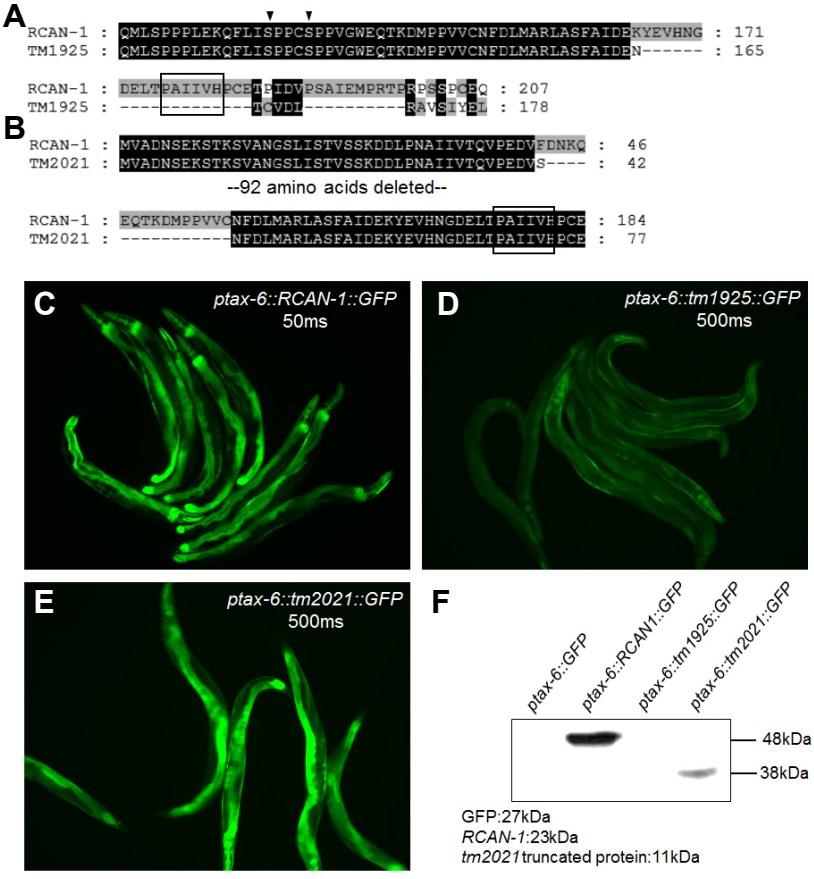

Fig. 5. *rcan-1* mutant alleles translation. Predicted translational products of *tm1925* (A) and *tm2021* (B) amino acid sequences were aligned with wild type RCAN-1 sequences. Arrow heads indicate phosphorylation sites, and the calcineurin binding motif is boxed, which is lost in the *tm1925* allele (Li et al., 2015). Fluorescence images from worms expressing genomic sequences of wild type *rcan-1* (C), *tm1925* (D), and *tm2021* (E) GFP fusion are shown at given exposures indicated in each image. (F) Transgenic worm western blot using polyclonal antibodies against RCAN-1. The wild type RCAN-1:GFP is detected at the expected 50 kDa migration, while *tm1925*: GFP was not detected at all. The *tm2021*: GFP migrated at the 38 kDa expected size, indicating that *tm2021* may produce the predicted 11 kDa truncated protein at low levels.

role in body size. Because *tm2021* showed normal body size, the truncated protein seemed to be sufficient to sustain normal growth rate. In the serotonin-mediated egg-laying behavior, those two *rcan-1* mutants lay slightly more eggs than wild types, indicating that *rcan-1* might be inhibitory for calcineurin in this behavior. However, those *rcan-1* mutations significantly suppressed *tax-6(jh107)gf*, while *tm2021* allele was more effective in suppression. These results suggest that *rcan-1* may be stimulatory to *tax-6* in certain circumstances.

rcan-1 is upregulated in the *tax-6(jh107)gf* background, presumably due to feedback induction via calcineurin activity (Li et al., 2015). *rcan-1* expression level seemed to be very critical in specific tissues, because *rcan-1* expression in AFD neurons rescued cryophilic *rcan-1* mutant phenotypes better than pan-neuronal expression (Li et al., 2015). Therefore, the truncated *tm2021* allele protein might accumulate more in *tax-6(jh107)gf* than in the transgenic line expressing GFP fusion. In addition, the elevated truncated protein containing PxIxIT and missing SP repeats may play a role in the potentiated suppression in *rcan-1(tm2021); tax-6(jh107)*. Finally, *rcan-1* lack significantly reversed the low *tax-6(ok2065)lf* egg-laying phenotype. Taken together, these data indicate that *rcan-1* is also required for normal egg-laying, and might stimulate calcineurin activity.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

ACKNOWLEDGMENTS

Strains are provided by the *Caenorhabditis* Genetic Center and National Bioresource Project (NBP-Japan). Kyung-Min Ko performed immunostaining of worms. This work was supported by the Basic Science Research Programs (no. 2015R1D1A1A 01059062) and (2015R1D1A1A01058179) through the National Research Foundation of Korea (NRF) funded by Korean Ministry of Science, ICT & Future Planning.

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