Calcineurin inhibition enhances *Caenorhabditis elegans* lifespan by defecation defects-mediated calorie restriction and nuclear hormone signaling

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Abstract

Calcineurin is a highly conserved calcium/calmodulin-dependent serine/threonine protein phosphatase with diverse functions. Inhibition of calcineurin is known to enhance *Caenorhabditis elegans* lifespan via multiple signaling pathways. Aiming to study the role of calcineurin in regulating innate immunity, we discover that calcineurin is required for the rhythmic defecation motor program (DMP) in *C. elegans*. Calcineurin inhibition leads to defects in the DMP, resulting in intestinal bloating, rapid colonization of the gut by bacteria, and increased susceptibility to bacterial infection. We demonstrate that intestinal bloating by calcineurin inhibition mimics calorie restriction that results in enhanced lifespan. The TFEB ortholog, HLH-30, is required for calcineurin inhibition-mediated lifespan enhancement by triggering lipolysis. Finally, we show that the nuclear hormone receptor, NHR-8, is upregulated by calcineurin inhibition and is required for increased lifespan. Our studies uncover a role for calcineurin in the *C. elegans* DMP and provide a new mechanism for calcineurin inhibition-mediated longevity extension.

Introduction

Interventions that enhance lifespan also impart resistance to multiple stresses (Johnson et al., 2001). Indeed, the positive correlation between improved stress resistance and enhanced lifespan has been exploited to identify long-lived mutants (Castro et al., 2004; Denzel et al., 2014; Johnson et al., 2001; Muñoz and Riddle, 2003; Wang et al., 2004). Among stress responses, innate immunity appears to be a crucial factor for enhanced lifespan (Campos et al., 2021; Fabian et al., 2021; Soo et al., 2023; Xia et al., 2019). However, the correlation between innate immunity and lifespan is not always positive. Interventions that alter lifespan may not modulate innate immunity, and vice-versa (Labed et al., 2018; Naim et al., 2021; Otarigho and Aballay, 2020; Sun et al., 2011). Some signaling pathways also establish a tradeoff between innate immunity and lifespan. Mutants that have improved immunity but reduced lifespan have been identified (Amrit et al., 2019; Otarigho and Aballay, 2021; Ren and Ambros, 2015). Conversely, mutants with enhanced lifespans but declined immune responses have also been discovered (Kawli et al., 2010). Moreover, the genetic pathways for lifespan and immunity could be uncoupled in mutants exhibiting enhanced lifespan and improved immune responses (Alper et al., 2010; Guerrero et al., 2021). Therefore, the relationship between lifespan and innate immunity appears to be complex and remains to be fully understood.

Calcineurin, a conserved protein from yeast to humans, is a calcium/calmodulin-dependent serine/threonine protein phosphatase that is involved in diverse cellular processes and signal transduction pathways (Chen et al., 2022; Hogan et al., 2003; Schulz and Yutzey, 2004; Ulengin-Talkish and Cyert, 2023). Dephosphorylation of substrate proteins by calcineurin affects several cellular pathways, including transcriptional signaling programs (Ulengin-Talkish and Cyert, 2023). Calcineurin regulates the activity of the transcription factors of the nuclear factor of activated T cells (NFAT) family (Hogan et al., 2003). Dephosphorylation of NFATs by calcineurin triggers their nuclear localization and activates immune responses in vertebrates (Herbst et al., 2015; Hogan et al., 2003; Vandewalle et al., 2014). In the nematode *Caenorhabditis elegans*, calcineurin regulates thermotaxis, body size, fertility, and lifespan (Bandyopadhyay et al., 2002; Dong et al., 2007; Kuhara et al., 2002; Lee et al., 2013). Knockdown of the catalytic subunit of calcineurin, *tax-6*, is known to enhance *C. elegans* lifespan via multiple pathways, including autophagy and CREB-regulated transcriptional

coactivators (CRTCs) (Dong et al., 2007; Dwivedi et al., 2009; Mair et al., 2011; Tao et al., 2013). However, the role of calcineurin in regulating *C. elegans* response to pathogen infections has not been studied. Because *C. elegans* lacks the NFAT transcription factors (Song et al., 2013), it will be intriguing to study how calcineurin inhibition impacts *C. elegans* innate immunity. These studies could also shed some light on the complex interplay between lifespan and immunity.

In this study, we examined the effect of calcineurin inhibition on *C. elegans* innate immunity. Surprisingly, we found that the knockdown of *tax-6* enhanced the susceptibility of *C. elegans* to bacterial infection, despite enhancing lifespan. We discovered that *tax-6* is required for the rhythmic defecation motor program (DMP). The knockdown of *tax-6* resulted in intestinal bloating due to defects in the DMP which enhanced susceptibility to bacterial infection by increasing gut colonization by bacteria. Intestinal bloating resulted in calorie restriction-like phenotypes, including reduced lipid levels, and led to increased lifespan. We discovered that the TFEB ortholog, HLH-30, is required for calcineurin inhibition-mediated lifespan extension by triggering lipolysis. Finally, we found that the nuclear hormone receptor, NHR-8, is upregulated by calcineurin inhibition and is required for increased lifespan. Our studies uncover a new mechanism for calcineurin inhibition-mediated longevity extension.

Results

Calcineurin knockdown enhances *C. elegans* susceptibility to *Pseudomonas aeruginosa* infection

To understand the role of calcineurin in *C. elegans* innate immune response, we studied the survival of a hypomorphic allele of *tax-6*, *tax-6(p675)*, on the pathogenic bacterium *P. aeruginosa* PA14. Surprisingly, the *tax-6(p675)* animals had a drastically reduced survival on *P. aeruginosa* compared to wild-type N2 animals (Figure 1A). Similarly, the knockdown of *tax-6* by RNA interference (RNAi) enhanced the susceptibility of animals to *P. aeruginosa* compared to that of control animals (Figure 1B). The effects of RNAi were specific to *tax-6*, as *tax-6(p675)* animals did not have a further increase in susceptibility to *P. aeruginosa* upon *tax-6* RNAi (Figure 1C). As reported earlier (Dong et al., 2007; Dwivedi et al., 2009; Mair et al., 2011; Tao et al., 2013), we confirmed that *tax-6* knockdown led to an increased lifespan on *E. coli* (Figure

1D). An earlier study demonstrated that calcineurin regulates cAMP response element-binding protein (CREB) and CRTCs to regulate lifespan in *C. elegans* (Mair et al., 2011). The knockdown of the CREB homolog-1 (*crh-1*) and *crtc-1* enhanced lifespan similar to the *tax-6* knockdown (Mair et al., 2011). We asked whether the knockdown of *crh-1* and *crtc-1* impacted *C. elegans* survival on *P. aeruginosa* comparable to the *tax-6* knockdown. Interestingly, the knockdown of *crh-1* and *crtc-1* did not affect the survival on *P. aeruginosa* as adversely as the knockdown of *tax-6* did (Figure S1). These results demonstrated that despite having an increased lifespan, *tax-6* knockdown animals have drastically enhanced susceptibility to *P. aeruginosa* infection by a mechanism likely independent of *crh-1* and *crtc-1*.

We observed enhanced matricidal hatching in *tax-6* knockdown animals on *P. aeruginosa*. Therefore, we asked whether the enhanced susceptibility of *tax-6* knockdown animals to *P. aeruginosa* was because of enhanced matricidal hatching. To this end, we studied the effects of *tax-6* knockdown in *fer-1(b232)* temperature-sensitive mutants. When grown at 25°C, *fer-1(b232)* animals have unfertilized oocytes (Argon and Ward, 1980), and therefore, these animals will lack matricidal hatching. As shown in Figure 1E, *fer-1(b232)* animals with unfertilized oocytes also had reduced survival on *P. aeruginosa* upon *tax-6* knockdown, indicating that the increased susceptibility upon *tax-6* knockdown is not because of enhanced matricidal hatching. The *fer-1(b232)* animals also showed enhanced lifespan on *E. coli* upon knockdown of *tax-6* (Figure 1F). These results suggested that the knockdown of *tax-6* led to increased lifespan and enhanced susceptibility to pathogen infection.

Next, we asked whether the enhanced susceptibility to bacterial infection upon *tax-6* knockdown was mediated by one or more of the established *C. elegans* innate immunity pathways, which include a MAP kinase pathway mediated by NSY-1/SEK-1/PMK-1 (Kim et al., 2002), the MLK-1/MEK-1/KGB-1 c-Jun kinase pathway (Kim et al., 2004), the TGF-β/DBL-1 pathway (Mallo et al., 2002), and the bZIP transcription factor ZIP-2 pathway (Estes et al., 2010). In mutants of all different immune pathways, the knockdown of *tax-6* further enhanced susceptibility to *P. aeruginosa* (Figure S2A-D). The enhanced susceptibility of *tax-6* knockdown animals to *P. aeruginosa* was also independent of the FOXO transcription factor DAF-16 (Figure S2E). Thus, the enhanced susceptibility to *P. aeruginosa* upon *tax-6* knockdown appears to be independent of established *C. elegans* innate immunity pathways.

Calcineurin is required for the *C. elegans* defecation motor program (DMP)

To understand why *tax-6* knockdown animals had enhanced susceptibility to *P. aeruginosa* infection, we studied the colonization of the intestine of *tax-6* RNAi animals by *P. aeruginosa*. Knockdown of *tax-6* led to enhanced intestinal colonization by *P. aeruginosa* compared to the control animals (Figure 2A, B). The *tax-6(p675)* animals also had increased intestinal colonization by *P. aeruginosa* (Figure 2C, D). Animals with bloated intestinal lumens exhibit enhanced bacterial colonization of the gut, an improved lifespan, and an increased susceptibility to pathogens (Kumar et al., 2019; Singh and Aballay, 2019a). Because *tax-6* knockdown animals displayed all of these phenotypes, we asked whether *tax-6* knockdown led to the bloating of the intestinal lumen. Indeed, we found that *tax-6* knockdown resulted in bloated intestinal lumens (Figure 2E, F).

Intestinal bloating could be an outcome of either defects in the pharyngeal pumping (Kumar et al., 2019) or defects in the defecation motor program (DMP) (Singh and Aballay, 2019a). Knockdown of *tax-6* did not affect the pharyngeal pumping rate (Figure S3A). We studied whether calcineurin was required for the DMP. The *C. elegans* DMP is a highly coordinated rhythmic behavior required for regular expulsion of the intestinal lumen contents with a frequency of about one cycle per minute. The DMP involves a series of muscle contractions involving posterior body muscle contraction, anterior body muscle contraction, and expulsion muscle contraction to release the lumen content (Thomas, 1990). We counted the number of expulsion events for 20 minutes per animal and found that *tax-6* knockdown animals had drastically reduced expulsion events (Figure 2G), leading to irregular DMP. The DMP-defect phenotype had a low penetrance in the *tax-6(p675)* animals. The *tax-6(p675)* animals exhibited both regular and irregular DMPs (Figure S3B). About 36% of the *tax-6(p675)* animals had irregular and slowed DMP, while the rest had a regular DMP (Figure 2H), suggesting that *tax-6(p675)* is a weak allele. Taken together, these results showed that calcineurin is required for the DMP, and its inhibition enhances susceptibility to *P. aeruginosa* by impeding the DMP.

Calcineurin inhibition enhances lifespan via DMP defects-mediated calorie restriction

Defects in *C. elegans* DMP result in reduced absorption of nutrients leading to diminished intestinal fat (Sheng et al., 2015). We asked whether *tax-6* knockdown resulted in reduced fat

levels. To this end, we carried out oil-red-O (ORO) staining of *tax-6* RNAi animals. Knockdown of *tax-6* resulted in drastically reduced fat levels (Figure 3A, B). Next, we tested whether the reduced fat levels in *tax-6* knockdown animals resulted in calorie restriction-like phenotypes. Calorie restriction is known to upregulate the expression of the FoxA transcription factor PHA-4 (Panowski et al., 2007). Knockdown of *tax-6* also resulted in the upregulation of *pha-4* expression levels (Figure 3C). Because calorie restriction enhances lifespan (Kaeberlein et al., 2006; Lakowski and Hekimi, 1998; Panowski et al., 2007), we asked whether calcineurin inhibition resulted in increased lifespan via calorie restriction. The knockdown of *tax-6* in the genetic model of calorie restriction, *eat-2(ad465)* (Lakowski and Hekimi, 1998), did not increase the lifespan (Figure 3D). These results suggested that calcineurin inhibition enhances lifespan via calorie restriction.

The mechanisms of lifespan enhancement by dietary or calorie restriction are complex and depend on several different downstream pathways (Chamoli et al., 2014; Greer and Brunet, 2009; Hansen et al., 2008). Further, different pathways are operational under different dietary regimens (Greer and Brunet, 2009). Therefore, we tested the role of different pathways linked with calorie restriction-mediated lifespan enhancement in increasing lifespan upon calcineurin inhibition. We studied the interactions of the AMP-activated kinase, *aak-2* (Greer and Brunet, 2009), the mTOR pathway, *raga-1* (Robida-Stubbs et al., 2012; Zhang et al., 2019), ribosomal S6 kinase, *rsks-1* (Chen et al., 2009; Selman et al., 2009; Zhang et al., 2019), the FOXO transcription factor, *daf-16* (Greer and Brunet, 2009), and the nuclear hormone receptor, *nhr-49* (Chamoli et al., 2014), with calcineurin inhibition in regulating lifespan. Mutants defective in *aak-2* appeared to have only a partial increase in lifespan upon calcineurin inhibition (Figure 3E). On the other hand, loss-of-function mutants of *raga-1*, *rsks-1*, *daf-16*, and *nhr-49* exhibited enhanced lifespan upon calcineurin inhibition (Figure 3F-I).

Calcineurin knockdown enhances lifespan via HLH-30 and NHR-8

To further explore longevity pathways regulated by calorie restriction that might enhance lifespan downstream of calcineurin inhibition, we studied the role of TFEB ortholog HLH-30. HLH-30 is required for starvation response and triggers lipid depletion under nutrient-deprived conditions (O'Rourke and Ruvkun, 2013). Knockdown of *tax-6* did not enhance the lifespan of *hlh-30(tm1978)* animals (Figure 4A), indicating that HLH-30 is required for increased lifespan

downstream of calcineurin inhibition. We studied whether HLH-30 affected fat depletion upon the knockdown of *tax-6*. While the ORO levels declined significantly in N2 animals upon knockdown of *tax-6* (Figure 3A, B), the ORO levels did not change in *hlh-30(tm1978)* animals upon *tax-6* knockdown (Figure 4B, C). Knockdown of *tax-6* resulted in defects in the DMP and intestinal bloating in *hlh-30(tm1978)* animals (Figure S4A-C). Therefore, the lack of fat depletion in *hlh-30(tm1978)* animals upon *tax-6* RNAi was not because of the absence of DMP defects. These results suggested that HLH-30-mediated lipid depletion enhanced lifespan upon calcineurin inhibition.

Steroid hormone signaling mediated by NHR-8 is known to regulate calorie restriction-mediated enhancement in lifespan (Thondamal et al., 2014). We tested whether NHR-8 was required for lifespan increment by calcineurin inhibition. Indeed, the knockdown of *tax-6* did not enhance the lifespan of *nhr-8(ok186)* animals (Figure 4D). While the ligands of NHR-8 remain poorly defined (Magner et al., 2013), the steroid hormones produced by the cytochrome P450 enzyme DAF-9 are known to enhance lifespan via NHR-8 under calorie restriction (Thondamal et al., 2014). Therefore, we tested whether DAF-9 mediated the enhanced lifespan downstream of calcineurin inhibition. We found that *tax-6* knockdown enhanced the lifespan of *daf-9(rh50)* animals (Figure 4E), indicating that DAF-9-produced steroid hormones were not required for lifespan increment by calcineurin inhibition. We asked whether NHR-8 affected the fat depletion of *tax-6* knockdown animals. The *nhr-8(ok186)* animals had drastically reduced fat levels upon the knockdown of *tax-6* (Figure 4F, G). These results indicated that NHR-8 worked downstream of or in parallel with fat depletion.

Because calorie restriction can also enhance the expression of genes that increase lifespan, such as *pha-4* (Panowski et al., 2007), we tested whether *tax-6* knockdown modulated the mRNA levels of *nhr-8*. Indeed, we observed that *tax-6* knockdown increased *nhr-8* mRNA levels significantly (Figure 4H). To test whether *tax-6* knockdown resulted in the upregulation of *nhr-8* because of intestinal bloating or independent of intestinal bloating, we studied *nhr-8* mRNA levels in other gene knockdown animals that had bloated intestinal lumens. We knocked down genes *flr-1*, *nhx-2*, and *pbo-1* as their inhibition is known to lead to defects in the DMP, resulting in intestinal bloating and enhanced lifespan (Singh and Aballay, 2019a). Knockdown of *flr-1*, *nhx-2*, and *pbo-1* also resulted in increased *nhr-8* mRNA levels (Figure 4H), indicating that intestinal bloating resulted in *nhr-8* upregulation. Therefore, calcineurin inhibition likely

enhanced lifespan via NHR-8 by upregulating its expression levels. Taken together, these results suggested that intestinal bloating caused by calcineurin inhibition enhances lifespan by lipolysis via HLH-30 and upregulating expression of NHR-8 (Figure 4I).

Discussion

We discovered that calcineurin is required for the *C. elegans* DMP. Calcineurin activity is regulated by intracellular calcium levels. Increased amounts of calcium ions activate the phosphatase activity of calcineurin via the binding of the calcium-sensing protein calmodulin to calcineurin (Klee et al., 1998). The rhythmic DMP cycle in *C. elegans* is known to be regulated by rhythmic calcium waves (Santo et al., 1999; Teramoto and Iwasaki, 2006). The calcium waves are regulated by the endoplasmic reticulum calcium channel, ITR-1, and mutations in the *itr-1* gene affect defecation by preventing cytoplasmic calcium release (Santo et al., 1999). It is likely that calcium waves regulate DMP via calcineurin activities. Indeed, calcineurin is expressed in the enteric muscles that are required for contractions for DMP (Lee et al., 2005). It is interesting to note that *tax-6* gain-of-function mutants are also known to have DMP defects (Lee et al., 2005). Therefore, optimum calcineurin activity appears to be crucial for maintaining a rhythmic DMP.

Calcineurin inhibition has been shown to extend *C. elegans* lifespan via multiple mechanisms (Dong et al., 2007; Dwivedi et al., 2009; Mair et al., 2011; Tao et al., 2013). Activation of autophagy has been shown to be one of the mechanisms of extended lifespan upon calcineurin inhibition (Dwivedi et al., 2009). We showed that defects in the DMP caused by calcineurin inhibition reduce lipid levels and mimic calorie restriction. Calorie restriction is known to induce autophagy (Morselli et al., 2010), and autophagy is required for calorie restriction-mediated lifespan extension (Hansen et al., 2008; Jia and Levine, 2007). Therefore, it is likely that reduced lipid levels because of intestinal bloating result in the activation of autophagy upon calcineurin inhibition. Importantly, the TFEB ortholog, HLH-30, is required for lipolysis and autophagy under starvation conditions (Lapierre et al., 2013; O'Rourke and Ruvkun, 2013). HLH-30 is also required for lifespan enhancement via autophagy and multiple longevity pathways (Lapierre et al., 2013; O'Rourke and Ruvkun, 2013). We observed that HLH-30 is also required for the increase in lifespan upon calcineurin inhibition. Because autophagy may regulate lifespan via multiple longevity pathways (Hansen et al., 2008; Lapierre

et al., 2013), this could explain why calcineurin inhibition enhances lifespan via multiple mechanisms.

Dietary restriction-mediated lifespan extensions are complex and context-dependent (Greer and Brunet, 2009). Multiple longevity pathways have been identified under different paradigms of dietary restriction (Chamoli et al., 2020, 2014; Chen et al., 2009; Greer and Brunet, 2009; Lapierre et al., 2013; Matai et al., 2019; Selman et al., 2009; Thondamal et al., 2014). We found that *tax-6* knockdown enhanced lifespan independent of multiple dietary restriction pathways, including *raga-1*, *rsks-1*, *daf-16*, and *nhr-49*. An earlier study identified that *tax-6* knockdown enhanced lifespan via NHR-49 (Burkewitz et al., 2015). We currently do not understand the reasons for this discrepancy. We identified that *tax-6* knockdown resulted in the upregulation of *nhr-8* mRNA levels, and *nhr-8* was required for the increased lifespan in *tax-6* knockdown animals. NHR-8 has been shown to mediate calorie restriction-dependent lifespan via the regulation of xenobiotic responses (Chamoli et al., 2014; Verma et al., 2018). Therefore, it is likely that NHR-8 increases lifespan upon calcineurin inhibition by activating xenobiotic response pathways.

Recent studies in different organisms have shown that gut bloating has profound effects on food-seeking behaviors, immunity, and lifespan (Duvall et al., 2019; Filipowicz et al., 2021; Kumar et al., 2019; Min et al., 2021; Singh and Aballay, 2019a, 2019b, 2019c). Several *C. elegans* mutants with defects in the DMP and bloated intestinal lumens are known to have dampened nutrient absorption, leading to reduced lipid deposition in the gut, mimicking calorie restriction (Sheng et al., 2015). It is possible that some of the effects of intestinal bloating on the host physiology are mediated by calorie restriction. Indeed, the neuropeptide Y receptors, which control a diverse set of behaviors, including appetite, are activated by gut bloating (Singh and Aballay, 2019c). Calorie restriction is known to induce neuropeptide Y, which might trigger feeding behaviors (Aveleira et al., 2015; de Rijke et al., 2005; Ferreira-Marques et al., 2016). The complete characterization of the physiological changes downstream of gut bloating may provide broad insights into the effects of gut physiology on behaviors, immunity, and lifespan.

Materials and Methods

Bacterial strains

The following bacterial strains were used: *Escherichia coli* OP50, *E. coli* HT115(DE3), *Pseudomonas aeruginosa* PA14, and *P. aeruginosa* PA14 expressing green fluorescent protein (*P. aeruginosa* PA14-GFP). The cultures for these bacteria were grown in Luria-Bertani (LB) broth at 37°C.

C. elegans strains and growth conditions

C. elegans hermaphrodites were maintained on nematode growth medium (NGM) plates seeded with *E. coli* OP50 at 20°C unless otherwise indicated. Bristol N2 hermaphrodites were used as the wild-type control unless otherwise indicated. The following strains were used in the study: PR675 *tax-6(p675)*, HH142 *fer-1(b232)*, DA465 *eat-2(ad465)*, CF1038 *daf-16(mu86)*, STE68 *nhr-49(nr2041)*, RB754 *aak-2(ok524)*, AE501 *nhr-8(ok186)*, VC222 *raga-1(ok386)*, RG1228 *daf-9(rh50)*, RB1206 *rsks-1(ok1255)*, KU25 *pmk-1(km25)*, KU21 *kgb-1(km21)*, NU3 *dbl-1(nk3)*, JIN1375 *hlh-30(tm1978)*, and VC3056 *zip-2(ok3730)*. Some of the strains were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN). The *fer-1(b232)* hermaphrodites were maintained on *E. coli* OP50 at 15°C and were grown at 25°C prior to *P. aeruginosa* killing assays and longevity assays to obtain animals with unfertilized oocytes.

RNA interference (RNAi)

RNAi was used to generate loss-of-function phenotypes by feeding nematodes *E. coli* strain HT115(DE3) expressing double-stranded RNA homologous to a target gene. RNAi was carried out as described previously (Gokul and Singh, 2022; Ravi et al., 2023). Briefly, *E. coli* with the appropriate vectors were grown in LB broth containing ampicillin (100 μg/mL) at 37°C overnight and plated onto NGM plates containing 100 μg/mL ampicillin and 3 mM isopropyl β-D-thiogalactoside (IPTG) (RNAi plates). RNAi-expressing bacteria were allowed to grow overnight at 37°C. Gravid adults were transferred to RNAi-expressing bacterial lawns and allowed to lay eggs for 2 hours. The gravid adults were removed, and the eggs were allowed to develop at 20°C to 1-day-old adults for subsequent assays. The RNAi clones were from the Ahringer RNAi library and were verified by sequencing.

C. elegans longevity assays

Lifespan assays were performed on RNAi plates containing *E. coli* HT115(DE3) with the empty vector control and *tax-6* RNAi clone in the presence of 50 µg/mL of 5-fluorodeoxyuridine (FUdR). Animals were synchronized on RNAi plates without FUdR and incubated at 20°C. At the late L4 larval stage, the animals were transferred onto the corresponding RNAi plates containing 50 µg/mL of FUdR and incubated at 20°C. For *fer-1(b232)* lifespan assays, the animals were synchronized on RNAi plates without FUdR and incubated at 25°C. The *fer-1(b232)* lifespan assays were carried out at 25°C without FUdR. Animals were scored every other day as live, dead, or gone. Animals that failed to display touch-provoked movement were scored as dead. Animals that crawled off the plates were censored. Experimental groups contained more than 80 animals per condition per replicate. Young adult animals were considered as day 0 for the lifespan analysis. Three independent experiments were performed.

C. elegans killing assays on _P. aeruginosa_ PA14

Bacterial cultures were prepared by inoculating individual bacterial colonies of *P. aeruginosa* into 2 mL of LB and growing them for 10-12 hours on a shaker at 37°C. Bacterial lawns were prepared by spreading 20 µL of the culture on the entire surface of 3.5-cm-diameter modified NGM agar plates (0.35% instead of 0.25% peptone). The plates were incubated at 37°C for 12-16 hours and then cooled to room temperature for at least 30 minutes before seeding with synchronized 1-day-old adult animals. The killing assays were performed at 25°C, and live animals were transferred daily to fresh plates. Animals were scored at times indicated and were considered dead when they failed to respond to touch.

P. aeruginosa-GFP colonization assay

Bacterial cultures were prepared by inoculating individual bacterial colonies of *P. aeruginosa*-GFP into 2 mL of LB and growing them for 10-12 hours on a shaker at 37°C. Bacterial lawns were prepared by spreading 20 µL of the culture on the entire surface of 3.5-cm-diameter modified NGM agar plates (0.35% instead of 0.25% peptone) containing 50 µg/mL of kanamycin. The plates were incubated at 37°C for 12 hours and then cooled to room temperature for at least 30 minutes before seeding with 1-day-old adult gravid adults. The assays were performed at 25°C. After 6 hours of incubation, the animals were transferred from *P.*

aeruginosa-GFP plates to fresh *E. coli* OP50 plates and visualized within 5 minutes under a fluorescence microscope.

Quantification of intestinal bacterial loads

The intestinal bacterial loads were quantified by measuring colony-forming units (CFU) as described earlier (Singh and Aballay, 2019b, 2019a) with appropriate modifications. Briefly, *P. aeruginosa*-GFP lawns were prepared as described above. After 6 hours of exposure, the animals were transferred from *P. aeruginosa*-GFP plates to the center of fresh *E. coli* OP50 plates thrice for 10 minutes each to eliminate bacteria stuck to their body. Afterward, ten animals/condition were transferred into 50 µL of PBS containing 0.01% triton X-100 and ground using glass beads. Serial dilutions of the lysates (10^1, 10^2, 10^3, 10^4) were seeded onto LB plates containing 50 µg/mL of kanamycin to select for *P. aeruginosa*-GFP cells and grown overnight at 37°C. Single colonies were counted the next day and represented as the number of bacterial cells or CFU per animal. At least three independent experiments were performed for each condition.

Fluorescence imaging

Fluorescence imaging was carried out as described previously (Gokul and Singh, 2022; Ravi et al., 2023). Briefly, the animals were anesthetized using an M9 salt solution containing 50 mM sodium azide and mounted onto 2% agarose pads. The animals were then visualized using a Nikon SMZ-1000 fluorescence stereomicroscope. The fluorescence intensity was quantified using Image J software.

Quantification of intestinal lumen bloating

The 1-day-old adult animals grown on the empty vector control and *tax-6* RNAi were anesthetized using an M9 salt solution containing 50 mM sodium azide, mounted onto 2% agarose pads, and imaged. The diameter of the intestinal lumen was measured using the Zeiss Zen Pro 2011 software. At least ten animals were used for each condition.

Measurement of defecation motor program (DMP) rate

Wild-type N2 and *tax-6(p675)* animals were synchronized and grown at 20°C on *E. coli* OP50 for 4 and 5 days, respectively, before measuring the DMP rate. For RNAi experiments, N2

worms were synchronized on EV and *tax-6* RNAi plates and grown for 4 days at 20°C before measuring the DMP rate. The DMP cycle length was scored by assessing the time between expulsions (which are preceded by posterior and anterior body wall muscle contraction and the contraction of enteric muscles in a normal regular pattern) (Thomas, 1990). The number of expulsion events in 20 minutes was measured for each worm. The DMP rate was recorded for 5-6 worms/condition.

Pharyngeal pumping assay

For the pharyngeal pumping assay, 1-day-old adult animals grown on the empty vector control and *tax-6* RNAi were used. The number of contractions of the terminal bulb of the pharynx was counted for 30 seconds per worm. The pumping rates for 20 worms were recorded for each condition.

Lipid staining

The oil-red-O (ORO) staining was carried out as described earlier (Lynn et al., 2015) with appropriate modification. Briefly, wild-type N2, *hlh-30(tm1978)*, and *nhr-8(ok186)* animals were synchronized on empty vector control and *tax-6* RNAi bacteria and grown at 20°C for 4 days. About 300-400 synchronized gravid adult worms from experimental plates were washed three times with 1X PBS plus 0.01% triton X-100 (PBST). To permeabilize the cuticle, 600 µL of 40% isopropanol was added to 100 µL of worm pellet and was rocked for three minutes. The animals were spun down at 500 rpm for 30 seconds, and then 600 µL of supernatant was removed. Subsequently, 600 µL of ORO working stock was added to the worm pellet to stain the worms and incubated at room temperature for 1 hour on a shaker. ORO working stock was freshly prepared by diluting the stock 0.5% ORO in isopropanol to 60% in water and rocked at room temperature for 2 hours, followed by the removal of debris with a 0.22 µm filter. Afterward, worm samples were pelleted, 600 µL of supernatant was removed, 600 µL of PBST was added, and the samples were rocked for 30 minutes at room temperature. After that, the worms were mounted on a 2% agarose pad and imaged. At least ten worms/condition were imaged. Three independent biological replicates were performed. The ORO intensity was quantified using Image J software.

RNA isolation and quantitative reverse transcription-PCR (qRT-PCR)

Animals were synchronized by egg laying. Approximately 40 N2 gravid adult animals were transferred to 9-cm RNAi plates seeded with *E. coli* HT115 expressing the appropriate vectors and allowed to lay eggs for 4-5 hours. The gravid adults were then removed, and the eggs were allowed to develop at 20°C for 96 hours. Subsequently, the animals were collected, washed with M9 buffer, and frozen in TRIzol reagent (Life Technologies, Carlsbad, CA). Total RNA was extracted using the RNeasy Plus Universal Kit (Qiagen, Netherlands). Residual genomic DNA was removed using TURBO DNase (Life Technologies, Carlsbad, CA). A total of 6 μg of total RNA was reverse-transcribed with random primers using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA).

qRT-PCR was conducted using the Applied Biosystems One-Step Real-time PCR protocol using SYBR Green fluorescence (Applied Biosystems) on an Applied Biosystems 7900HT real-time PCR machine in 96-well-plate format. Twenty-five-microliter reactions were analyzed as outlined by the manufacturer (Applied Biosystems). The relative fold-changes of the transcripts were calculated using the comparative $CT(2^{-\Delta\Delta CT})$ method and normalized to pan-actin (*act-1, -3, -4*) as described earlier (Singh and Aballay, 2019a, 2017). The cycle thresholds of the amplification were determined using StepOnePlus software (Applied Biosystems). All samples were run in triplicate. The primer sequences are shown in Table S1.

Quantification and statistical analysis

The statistical analysis was performed with Prism 8 (GraphPad). All error bars represent mean±standard deviation (SD). The two-sample *t*-test was used when needed, and the data were judged to be statistically significant when $p < 0.05$. In the figures, asterisks (*) denote statistical significance as follows: *, $p<0.05$, **, $p<0.001$, ***, $p<0.0001$, as compared with the appropriate controls. The Kaplan-Meier method was used to calculate the survival fractions, and statistical significance between survival curves was determined using the log-rank test. All experiments were performed in triplicate.

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Author contributions

Priyanka Das: Conceptualization, Data curation, Formal analysis, Investigation, Methodology

Alejandro Aballay: Conceptualization, Visualization, Supervision

Jogender Singh: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Visualization, Supervision, Writing—original draft, Writing – review and editing

Competing interest: The authors declare that they have no competing interest.

Data Availability: All data generated or analyzed during this study are included in the manuscript and supporting files.

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Figures

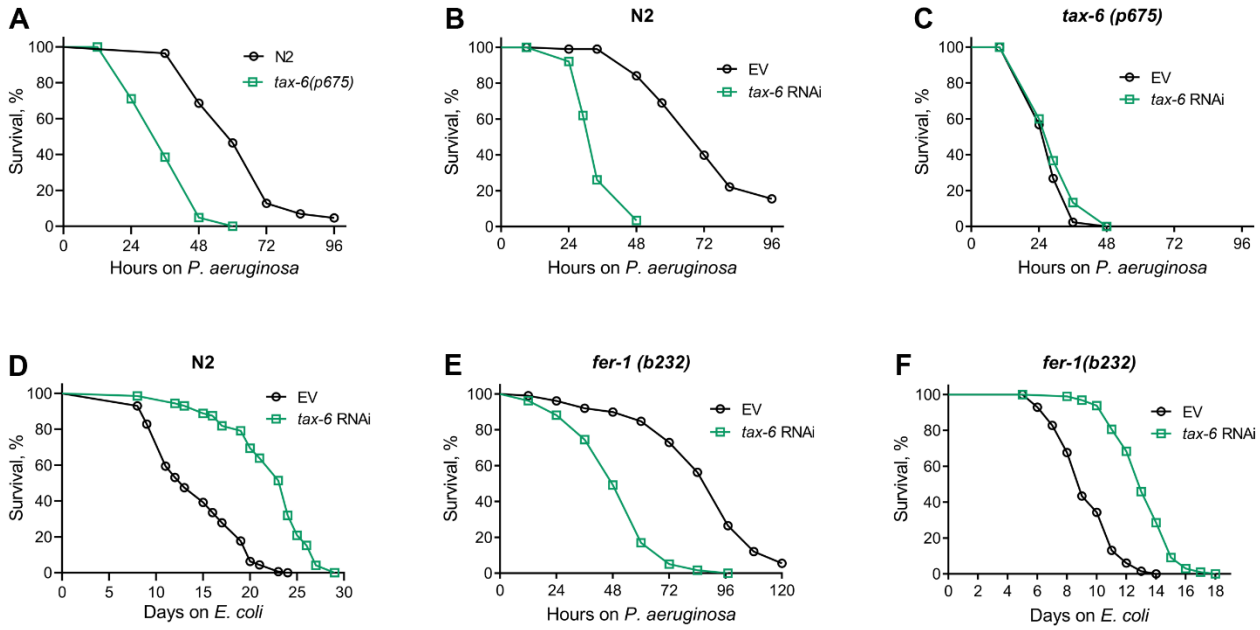

Figure 1: Calcineurin knockdown enhances *C. elegans* susceptibility to *Pseudomonas aeruginosa*

(A) Representative survival plots of N2 and *tax-6(p675)* animals on *P. aeruginosa* PA14 at 25°C. The animals were grown on *E. coli* OP50 at 20°C until 1-day-old adults before transferring to *P. aeruginosa* PA14 at 25°C. $p<0.001$

(B) Representative survival plots of N2 animals on *P. aeruginosa* PA14 at 25°C after treatment with the empty vector (EV) control and *tax-6* RNAi. $p<0.001$.

(C) Representative survival plots of *tax-6(p675)* animals on *P. aeruginosa* PA14 at 25°C after treatment with the EV control and *tax-6* RNAi. n.s., nonsignificant.

(D) Representative survival plots of N2 animals grown on bacteria for RNAi against *tax-6* along with the EV control at 20°C. Day 0 represents young adults. $p<0.001$.

(E) Representative survival plots of *fer-1(b232)* animals on *P. aeruginosa* PA14 at 25°C after treatment with the EV control and *tax-6* RNAi at 25°C. $p<0.001$.

(F) Representative survival plots of *fer-1(b232)* animals grown on bacteria for RNAi against *tax-6* along with the EV control at 25°C. The animals were developed at 25°C. Day 0 represents young adults. $p<0.001$.

For all panels, n = 3 biological replicates; animals per condition per replicate > 90.

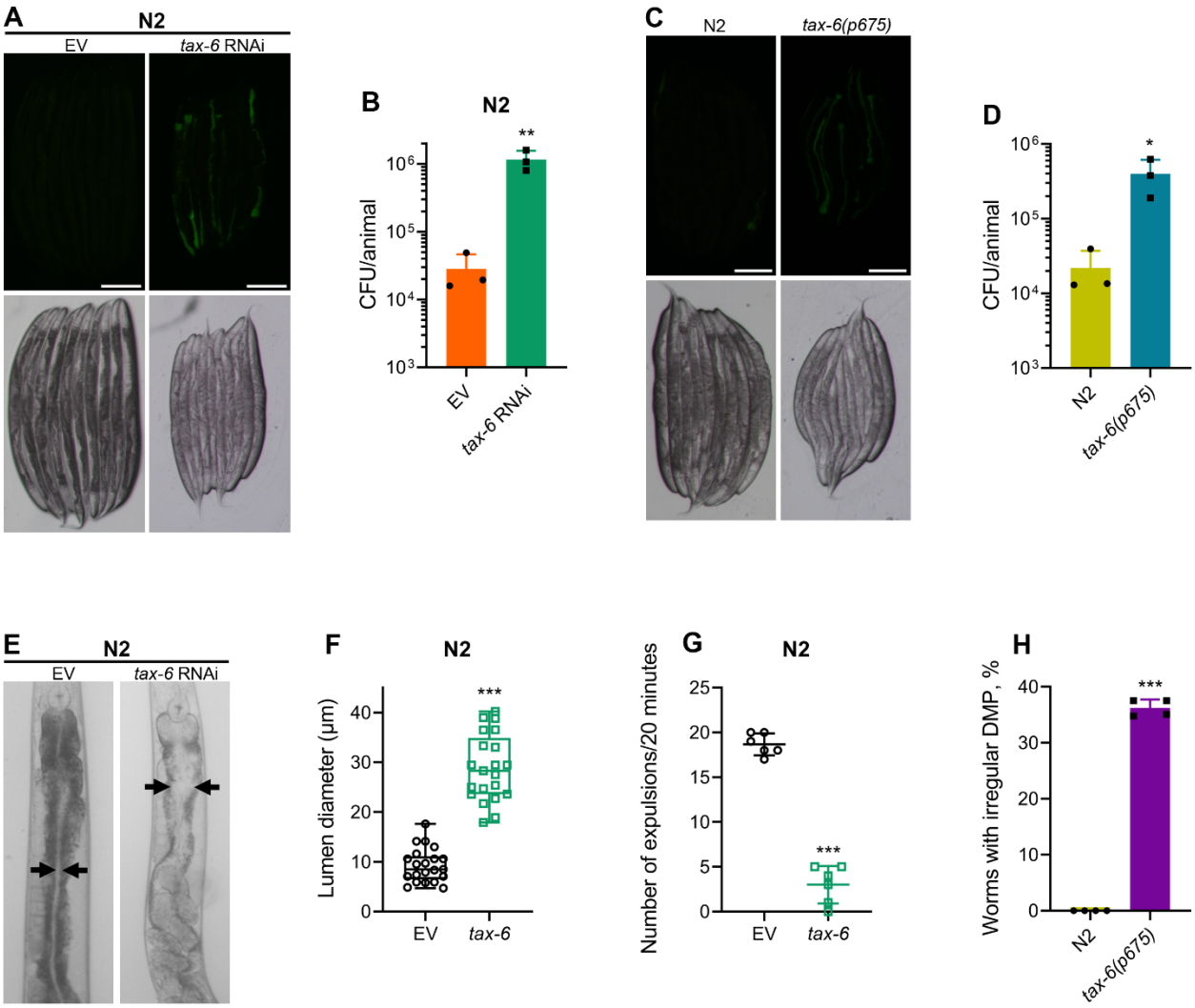

Figure 2: Calcineurin is required for the *C. elegans* defecation motor program (DMP)

(A) Representative fluorescence (top) and the corresponding brightfield (bottom) images of N2 animals incubated on *P. aeruginosa*-GFP for 6 hours at 25°C after growth on the empty vector (EV) control and *tax-6* RNAi bacteria.

(B) Colony-forming units (CFU) per animal of N2 worms incubated on *P. aeruginosa*-GFP for 6 hours at 25°C after growth on the EV control and *tax-6* RNAi bacteria. **$p < 0.01$ via the *t*-test (n = 3 biological replicates).

(C) Representative fluorescence (top) and the corresponding brightfield (bottom) images of N2 and *tax-6(p675)* animals incubated on *P. aeruginosa*-GFP for 6 hours at 25°C after growth on *E. coli* OP50 at 20°C.

(D) CFU per animal of N2 and *tax-6(p675)* worms incubated on *P. aeruginosa*-GFP for 6 hours at 25°C after growth on *E. coli* OP50 at 20°C. *$p < 0.05$ via the *t*-test (n = 3 biological replicates).

(E) Representative photomicrographs of N2 animals grown on the EV control and *tax-6* RNAi bacteria at 20°C until 1-day-old adults. Arrows point to the border of the intestinal lumen.

(F) Quantification of the diameter of the intestinal lumen of N2 animals grown on the EV control and *tax-6* RNAi bacteria at 20°C until 1-day-old adults. ***$p<0.001$ via the *t*-test (n = 21 worms each).

(G) The number of expulsion events observed in 20 minutes in 1-day-old adult N2 animals grown on the EV control and *tax-6* RNAi bacteria at 20°C. ***$p<0.001$ via the *t*-test (n = 6 worms each).

(H) Percent of 1-day-old adult worms having irregular DMP. ***$p<0.001$ via the *t*-test (n = 4 biological replicates).

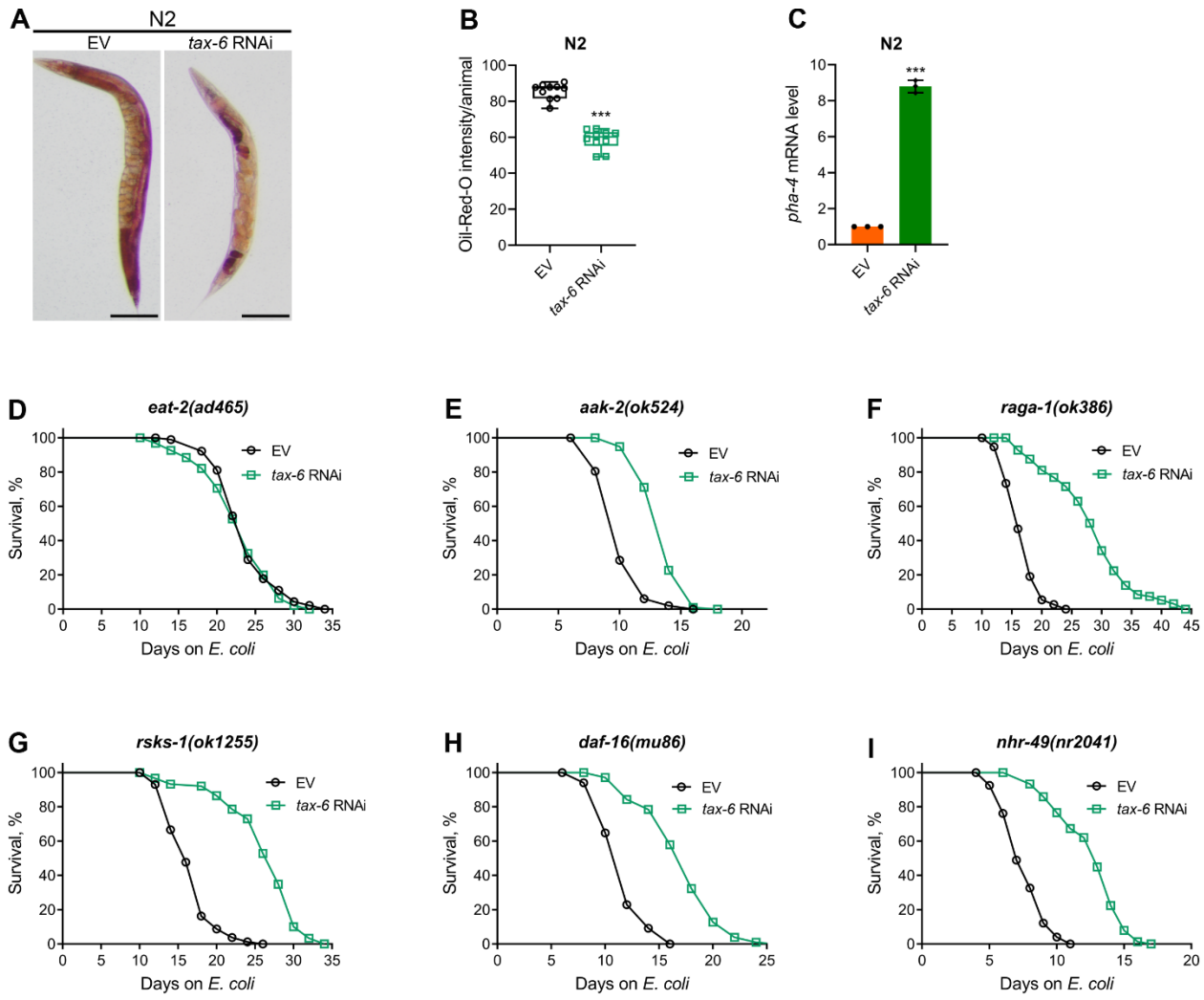

Figure 3: Calcineurin knockdown enhances lifespan via DMP defects-mediated calorie restriction

(A) Representative photomicrographs of oil-red-O (ORO) stained 1-day-old adult N2 animals grown on the empty vector (EV) control and *tax-6* RNAi bacteria at 20°C. Scale bar = 200 µm.

(B) Quantification of ORO intensity per animal of 1-day-old adult N2 animals grown on the EV control and *tax-6* RNAi bacteria at 20°C. ***$p<0.001$ via the *t*-test (n = 10 worms each).

(C) Quantitative reverse transcription-PCR (qRT-PCR) for *pha-4* mRNA levels in N2 animals grown on the EV control and *tax-6* RNAi bacteria at 20°C until 1-day-old adults. ***$p < 0.001$ (n = 3 biological replicates).

(D) Representative survival plots of *eat-2(ad465)* animals grown on the bacteria for RNAi against *tax-6* along with the EV control at 20°C. Day 0 represents young adults. n.s., nonsignificant (n = 3 biological replicates; animals per condition per replicate > 90).
(E-I) Representative survival plots of *aak-2(ok524)* (E), *raga-1(ok386)* (F), *rsks-1(ok1255)* (G), *daf-16(mu86)* (H), and *nhr-49(nr2041)* (I) animals grown on the bacteria for RNAi against *tax-6* along with the EV control at 20°C. Day 0 represents young adults. $p<0.001$ for all the plots (n = 3 biological replicates; animals per condition per replicate > 80).

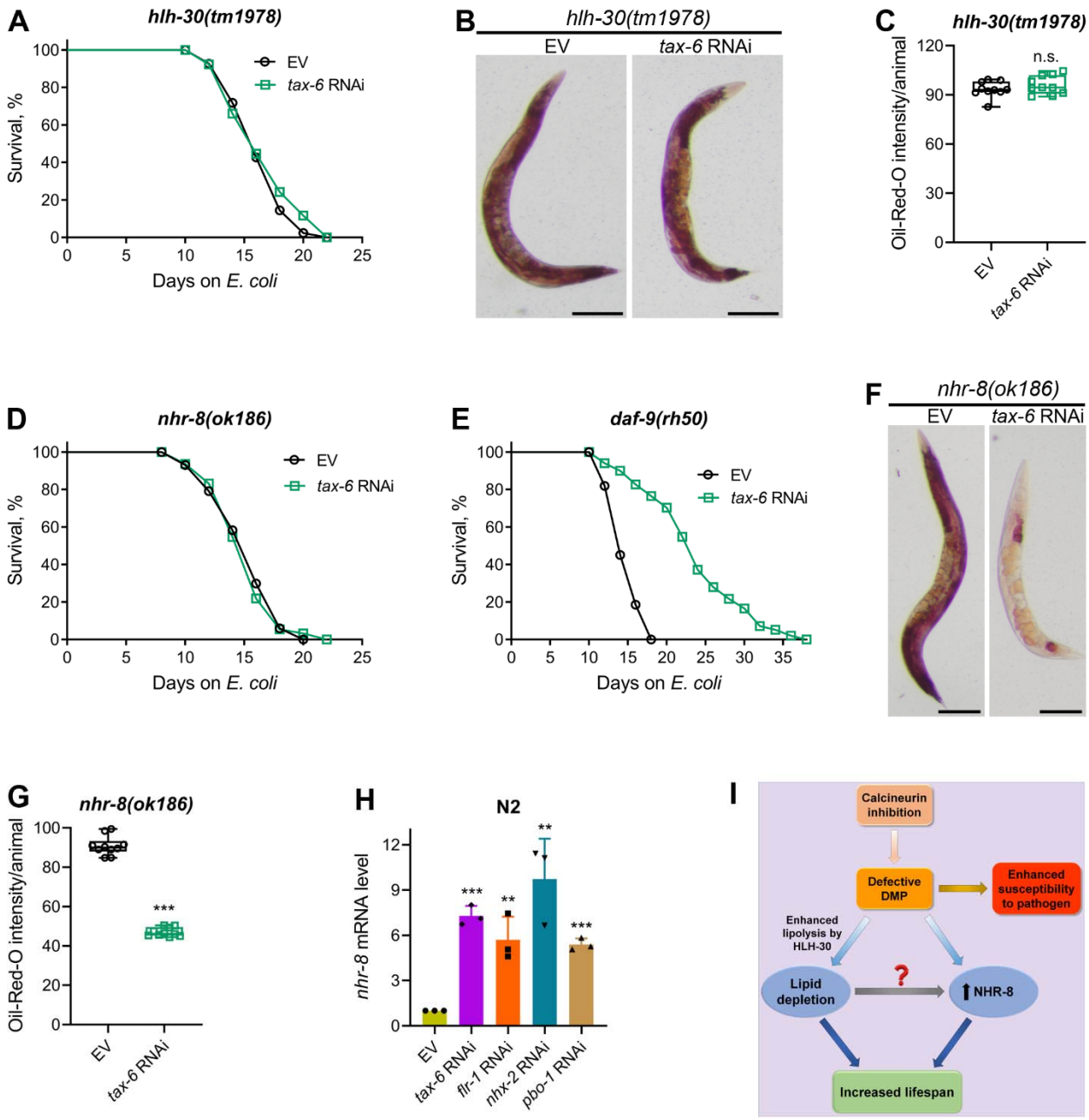

Figure 4: Calcineurin inhibition enhances lifespan via HLH-30 and NHR-8

(A) Representative survival plots of *hlh-30(tm1978)* animals grown on the bacteria for RNAi against *tax-6* along with the empty vector (EV) control at 20°C. Day 0 represents young adults. n.s., nonsignificant (n = 3 biological replicates; animals per condition per replicate > 90).

(B) Representative photomicrographs of oil-red-O (ORO) stained 1-day-old adult *hlh-30(tm1978)* animals grown on the EV control and *tax-6* RNAi bacteria at 20°C. Scale bar = 200 µm.

(C) Quantification of ORO intensity per animal of 1-day-old adult *hlh-30(tm1978)* animals grown on the EV control and *tax-6* RNAi bacteria at 20°C. n.s., nonsignificant via the *t*-test (n = 10 worms each).

(D) Representative survival plots of *nhr-8(ok186)* animals grown on the bacteria for RNAi against *tax-6* along with the EV control at 20°C. Day 0 represents young adults. n.s., nonsignificant (n = 3 biological replicates; animals per condition per replicate > 90).

(E) Representative survival plots of *daf-9(rh50)* animals grown on the bacteria for RNAi against *tax-6* along with the EV control at 20°C. Day 0 represents young adults. $p<0.001$ (n = 3 biological replicates; animals per condition per replicate > 80).

(F) Representative photomicrographs of ORO stained 1-day-old adult *nhr-8(ok186)* animals grown on the EV control and *tax-6* RNAi bacteria at 20°C. Scale bar = 200 µm.

(G) Quantification of ORO intensity per animal of 1-day-old adult *nhr-8(ok186)* animals grown on the EV control and *tax-6* RNAi bacteria at 20°C. ***$p<0.001$ via the *t*-test (n = 10 worms each).

(H) Quantitative reverse transcription-PCR (qRT-PCR) for *nhr-8* mRNA levels in N2 animals grown on the EV control, *tax-6*, *flr-1*, *nhx-2*, and *pbo-1* RNAi bacteria at 20°C until 1-day-old adults. ***$p < 0.001$ via the *t*-test (n = 3 biological replicates).

(I) Model depicting the mechanism of increased lifespan and enhanced susceptibility to pathogen upon inhibition of calcineurin via the defects in the defecation motor program (DMP).

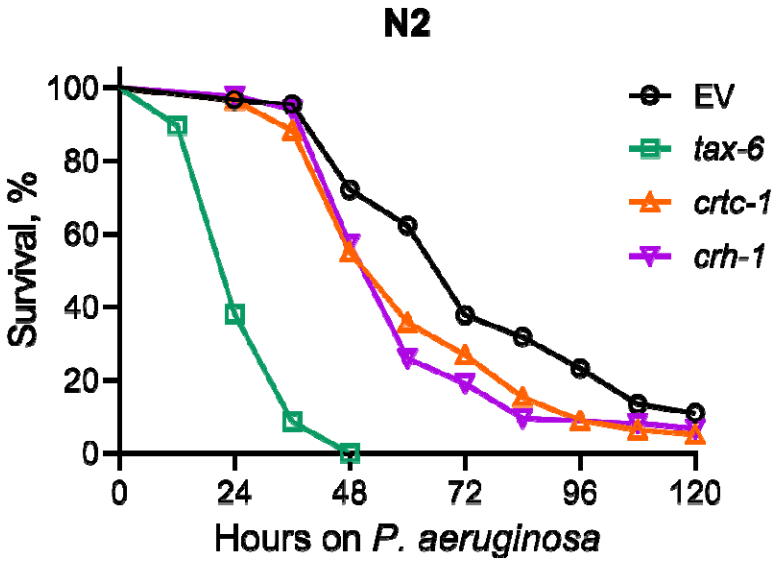

Figure S1: Calcineurin knockdown enhances *C. elegans* susceptibility to *Pseudomonas aeruginosa*

Representative survival plots of N2 animals on *P. aeruginosa* PA14 at 25°C after treatment with the empty vector (EV) control, *tax-6*, *crtc-1*, and *crh-1* RNAi. . $p<0.001$ for *tax-6* and $p<0.01$ for *crtc-1* and *crh-1* compared to EV (n = 3 biological replicates; animals per condition per replicate > 90).

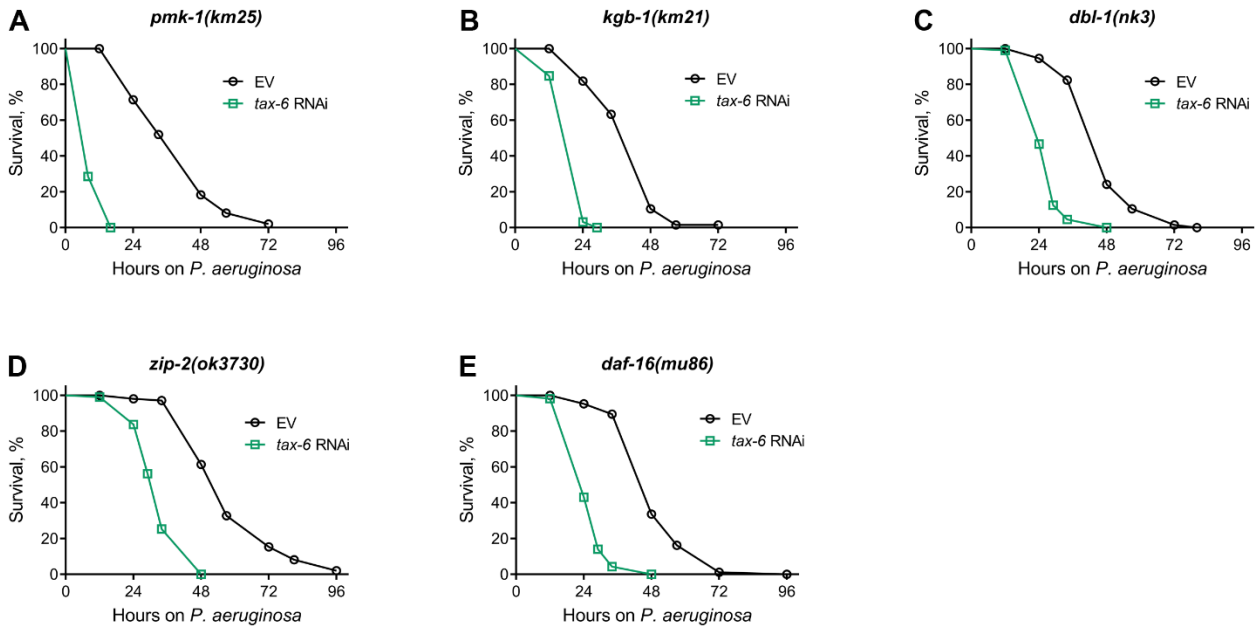

Figure S2: Calcineurin inhibition enhances susceptibility to *P. aeruginosa* independent of known immunity pathways

(A-E) Representative survival plots of *pmk-1(km25)* (A), *kgb-1(km21)* (B), *dbl-1(nk3)* (C), *zip-2(ok3730)* (D), and *daf-16(mu86)* (E) animals on *P. aeruginosa* PA14 at 25°C after treatment with the empty vector (EV) control and *tax-6* RNAi. $p<0.001$ for all the plots (n = 3 biological replicates; animals per condition per replicate > 90).

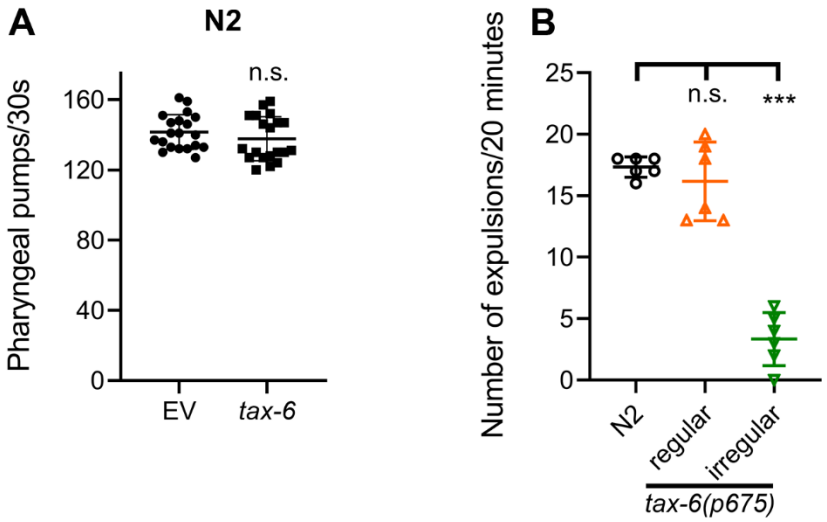

Figure S3: Calcineurin inhibition leads to defects in the defecation motor program (DMP) without affecting the pharyngeal pumping rate

(A) Pharyngeal pumps per 30 seconds of 1-day-old adult N2 animals grown on the empty vector (EV) control and *tax-6* RNAi bacteria at 20°C. n.s., nonsignificant via the *t*-test (n = 20 worms each).

(B) The number of expulsion events observed in 20 minutes in 1-day-old adult N2 and regular and irregular *tax-6(p675)* animals grown on *E. coli* OP50 at 20°C. ***p<0.001, n.s., nonsignificant via the *t*-test (n = 6 worms each).

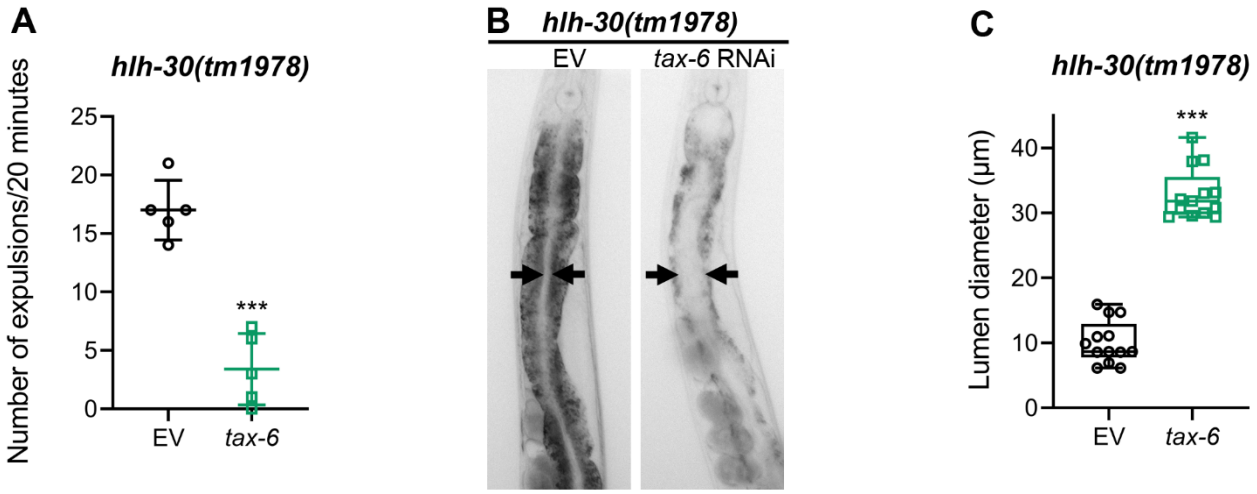

Figure S4: Calcineurin inhibition leads to defects in the defecation motor program (DMP) in *hlh-30(tm1978)* animals

(A) The number of expulsion events observed in 20 minutes in 1-day-old adult *hlh-30(tm1978)* animals grown on the empty vector (EV) control and *tax-6* RNAi bacteria at 20°C. ***p<0.001 via the *t*-test (n = 5 worms each).

(B) Representative photomicrographs of *hlh-30(tm1978)* animals grown on the EV control and *tax-6* RNAi bacteria at 20°C until 1-day-old adults. Arrows point to the border of the intestinal lumen.

(C) Quantification of the diameter of the intestinal lumen of *hlh-30(tm1978)* animals grown on the EV control and *tax-6* RNAi bacteria at 20°C until 1-day-old adults. ***p<0.001 via the *t*-test (n = 13 worms each).

Table S1: Primers used in the study

Primer for quantitative reverse transcription-PCR in *C. elegans*		
Gene name	Forward primer sequence (5'-3')	Reverse primer sequence (5’-3’)
Pan-act	TCGGTATGGGACAGAAGGAC	CATCCCAGTTGGTGACGATA
pha-4	CAAAGAGGAGCCAGAGTCGG	TGTTTCTGCTCGCGTTTTCG
nhr-8	CTACACAGTTTCTCCGGCGT	GCCATTTGGGCCATAACACC