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Integration of diet, stress, and hunger signals by the SIK kinase to coordinate gene expression, development and behavior

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ABSTRACT

Animals change their behavior and development depending on environmental and internal signals, but the underlying mechanisms are poorly understood. We postulate that the integration of diet, stress, and hunger signals by the SIK kinase acting in a subset of sensory neurons, coordinates gene expression, development, and behavior. Understanding the mechanisms of SIK is therefore critical to understand the ways in which environmental signals like food or stress, and internal state signals such as hunger or fatigue, act to modulate gene expression, development, and behavior. **Chapter 1** describes the role of salt-inducible kinases (SIKs) and their downstream targets as a signal integrator of external food and stress cues and internal signals such as hunger and fatigue, to coordinate development, gene expression, and behavior. With only one SIK isoform, KIN-29, the nematode *Caenorhabditis elegans* lends itself as an attractive model to study SIK signaling. In **Chapter 2**, using the nematode *C. elegans*, I show that the KIN-29/SIK dependent chemoreceptor gene *srh-234* is regulated by dietary vitamin B12 via the myocyte enhancer transcription factor 2 (MEF-2) identifying a mechanism in which animals may fine tune their chemoreceptor gene expression to allow them to modify their behavioral responses. **Chapter 3** identifies a role for KIN-29/SIK in aging and longevity and how it regulates lifespan via the class II histone-deacetylase HDA-4. Lastly, **Chapter 4** explores the role of food and the downstream phosphorylation target

of KIN-29/SIK, the CREB-regulated transcription cofactor 1 (CRTC-1) in the regulation of stress-induced sleep.

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1. INTRODUCTION

Animals receive environmental and internal state signals that must be coordinated to elicit appropriate behavioral responses. For instance, organisms must synthesize both external food availability signals and internal state signals such as hunger, to ultimately alter their behavior. Similarly, external stressors as a result of noxious environmental conditions or infection can lead to changes in internal state causing a protective sleep response. However, the underlying mechanisms of these behavioral responses is poorly understood. An animal's ability to respond to their environment relies on information integration by the nervous system. These decision-making centers must regulate behavioral responses accordingly based also on an animal's internal physiological state such as hunger, reproductive drive, stress or risk level (Gillette, Huang, Hatcher, & Moroz, 2000; Schall, 2001). Inappropriate signal interpretation can be inefficient or even lethal for an organism's survival. The goal of this work is to further the understanding of the underlying mechanisms of how animals synchronously process both environmental cues and internal physiological states

to modify gene expression and behavior. Using *C. elegans* as a model, my central hypothesis states that environmental and internal diet, stress and hunger signals converge in a subset of sensory neurons where the SIK kinase KIN-29 acts, to coordinates gene expression, development, and behavior.

2. Animal-environment interactions

Animals are constantly faced with internal and external signals that must be processed to appropriately interact with their environments. Simplified, the relationship between animal and environment consists of sensory input and behavioral output. As a result, animals respond to these signals and change their behaviors accordingly, but the underlying mechanisms remain uncertain. One of the first steps of animal-environment interactions is an animal's ability to sense its external surroundings. The perception of sensory stimuli allows animals to find resources and avoid risk to adapt and exist in their environments (Liang & Brinkman, 2022; Zylberberg & Deweese, 2011).

In addition to receiving external stimuli from the environment, animals must process and respond to internal physiological state signals. An animal's internal state also exerts control over behavioral outputs. The drive to feed, rest, fight, or reproduce are controlled by the changes in internal state (Berridge, 2004; Miczek et al., 2007). For example, in chapter 2, I explore the effects of diet as an external cue and its role in modulating chemosensory genes by altering the internal state in a single sensory neuron of the nematode *C. elegans.* The

combination of internal hunger signals and external predator odor cues changes an animal's food seeking behavior (Aimé et al., 2007; Edgecomb, Harth, & Schneiderman, 1994). In another instance, the balancing of foraging and safety drives during female pregnancy affects offspring care and breeding activity (Miller, Barr, Krawczyk, & Covey, 2016; Rodríguez-Saltos, Lyons, Sockman, & Maney, 2018). Additionally, the deprivation or perturbation of an organism's basic needs like food, sleep, and safety (Maslow, 1943) can quickly and robustly alter internal state and lead to appropriate behavioral changes (Kanwal et al., 2021). This is illustrated in chapter 3, where I describe that environmental stressors like extreme heat or UV irradiation on an organism can alter internal physiology and result in sleepiness or fatigue. In this situation, the drive to sleep must override other functions for the welfare of the organism. The cost-benefit analysis by the nervous system relies on multimodal signal integration of external environmental signals and internal state. The integration of external and internal signals allows animals to optimize their behavior. This begs the question, what are the mechanisms behind how animals detect, process, and integrate and environmental information?

3. SIK family and their functions

One of the most studied protein kinases and key sensor of cellular energy is the AMP-activated protein kinase (AMPK). A subfamily AMPKs, salt-inducible kinases (SIKs), were first identified over 20 years ago in the adrenal glands of

rats fed a high-salt diet (Z. Wang, Takemori, Halder, Nonaka, & Okamoto, 1999). While SIKs have not appeared to be involved in energy sensing and regulation, they have been shown to be major regulators of gene expression (Sakamoto, Bultot, & Göransson, 2018; Wein, Foretz, Fisher, Xavier, & Kronenberg, 2018). The SIK family is highly conserved, broadly expressed, and contains three kinase members (SIK1/2/3). SIK1 is expressed abundantly in the adrenal cortex (Katoh et al., 2004; Lin et al., 2001; Z. Wang et al., 1999), followed by adipose (Horike et al., 2003), and neural tissues (Feldman et al., 2000). In addition to excess dietary salt, SIK1 expression is regulated by adrenal signaling (Lin et al., 2001), gluconeogenic signaling (Koo et al., 2005), cAMP signaling (Stewart, Akhmedov, Robb, Leiter, & Berdeaux, 2013), and circadian rhythms (Jagannath et al., 2013). SIK2 mainly expressed in adipose tissues in mammals may play a vital role in adipocyte differentiation (Horike et al., 2003; Katoh et al., 2004). SIK2 is also critical in the early phase regulation of insulin-signaling in adipocytes (Katoh et al., 2004). Lastly, SIK3 is highly ubiquitous and abundant in neural tissue (Katoh et al., 2004; Wein et al., 2018) and has been shown to play a role in sleep-related neurotransmission (Z. Wang et al., 2018).

3.1. Upstream regulators of SIKs

SIKs are governed by several upstream regulators including the liver kinase B1 (LKB1), protein kinase A (PKA), post-translational modifications like acetylation. Like other AMPK family members, SIKs are phosphorylated by LKB1

(Lizcano et al., 2004). LKB1 is constitutively active meaning SIKs are therefore constitutively phosphorylated and tonically active in cells (Sakamoto et al., 2018). The LKB1 phosphorylation site is relatively conserved across all SIK isoforms but certain binding domains are more similar between SIK2 and SIK3 compared to SIK1 (Katoh et al., 2004; Sakamoto et al., 2018). The relationship between LKB1 and SIKs is most known for their role in mediating anti-tumorigenesis effects in various cancers (Murray et al., 2019; Patel et al., 2014). Much of the work done on LKB1 and SIKs has been in understanding role of SIKs in the mediation of LBK1 tumor suppressor functions (Eichner et al., 2019; Hollstein et al., 2019; Murray et al., 2019). Additionally, the LKB1-SIK axis plays an important role in regulation of hepatic lipid metabolism but degree of meditation and involvement is unclear. It has also been found that in the absence of LKB1, SIK1 is still activated suggesting residual activation by other regulators like CaMK (Murray et al., 2019). Another upstream regulator of SIK is PKA, which has been shown to have an active role in cancers though not considered an oncogene (Moody et al., 2015; Porter, Dwyer-Nield, & Malkinson, 2001; Shaikh et al., 2012). PKAs can directly phosphorylate SIKs to promote nuclear translocation but this does not alter the intrinsic kinase activity of SIKs (Henriksson et al., 2015). Loss of PKA promotes the protein stability of SIK and accelerates SIK expression (Stewart et al., 2013). PKA therefore would act as a negative regulator of SIKs that competes with LKB1 in ruling SIK signaling (Sun, Jiang, Li, & Guo, 2020). Other post-translational modifications beyond phosphorylation may also play a role in

the control of SIK activity. Ultimately, the specific mechanisms of these regulators must be further explored to understand the upstream regulation of SIKs.

3.2. Downstream substrates of SIKs

A major role of all three SIKs is to alter gene expression in response to extra-and intracellular signals (Wein et al., 2018). SIKs have many downstream substrates, the most well-known being class IIa histone deacetylases (HDAC4/5/7/9) (Haberland, Montgomery, & Olson, 2009), and CREB-regulated transcription cofactors (CRTC1/2/3) (Altarejos & Montminy, 2011). SIKs phosphorylate these targets and cause them to be sequestered by 14-3-3 chaperone proteins and remain in the cytoplasm but upon dephosphorylation, they are translocated to the nucleus to regulate gene expression (**Figure 1**) (Altarejos & Montminy, 2011; Haberland et al., 2009; Wein et al., 2018).

3.2a. Class IIa histone deacetylases

One of the best studied substrates of SIKs are class IIa HDACs (Haberland et al., 2009). Class IIa HDACs consist of HDAC4, 5, 7, and 9. This class of HDACs have somewhat restricted patterns of expression and are enriched in muscles and heart (HDAC5/9) (Chang et al., 2004; C. L. Zhang et al., 2002), brain and skeleton (HDAC4) (Vega et al., 2004), and endothelial cells (HDAC7) (Chang et al., 2006). These HDACs interact with a number of transcription factors including the myocyte enhancer factor MEF2 which plays an

essential role in regulating gene expression in response to environmental signals (Lu, McKinsey, Zhang, & Olson, 2000; van der Linden, Nolan, & Sengupta, 2007). SIK-mediated phosphorylation of HDAC retains HDAC in the cytoplasm thus repressing MEF2-dependent transcription (Chen, Zhao, & Zhao, 2015; Darling & Cohen, 2021; Haberland et al., 2009). Localization of HDACs and their subsequent activity is dependent on SIK-mediated phosphorylation (Haberland et al., 2009). This class of HDACs also play an essential role in activating the transcription factor FOXO involved in longevity downstream of insulin signaling (Guo et al., 1999; Murphy et al., 2003). The translocation of HDACs to the nucleus leads to regulation of FOXO and glucose homeostasis (Driver, Lamb, Wyner, & Raizen, 2013; Mihaylova et al., 2011). HDACs recruit additional players to deacetylate FOXO transcription factors and activate the transcription of gluconeogenic gene promoters (Driver et al., 2013; Mihaylova et al., 2011). However, the precise mechanisms through which HDACs modulate transcription is not well understood.

3.2b. CREB-regulated transcription co-factors

The discovery of CRTCs as a direct substrate of SIKs was a key finding in understanding the relationship of SIKs and the important transcription factor CREB, a major regulator of cellular responses. There are three CRTCs that are transcriptional coactivators required for CREB-dependent transcription (Iourgenko et al., 2003). Like SIKs, all three CRTC isoforms are widely expressed and while structurally and functionally related, have distinct

distribution patterns across tissues (Conkright et al., 2003). CRTC2 and CRTC3 and highly expressed in the liver while CRTC1 is found to be abundant in the brain and neural tissues (Conkright et al., 2003; Gao, Tang, Cheng, Chan, & Jin, 2018). CRTCs like SIKs are involved in the regulation of a variety of signaling pathways. CRTC2 has a role in lipid metabolism (Han et al., 2015; Y. Li et al., 2017) and CRTC3 is required for the regulation of hormones in stress response (Jurek et al., 2015). Both CRTC2 and CRTC3 are a found to be involved in the regulation of gluconeogenesis and energy homeostasis (Patel et al., 2014; Shan et al., 2016) but CRTC1 has been demonstrated to have a function in neuronal processes. In particular, CRTC1 is involved in learning and memory (Parra-Damas et al., 2017; Uchida et al., 2017), neuronal support and growth (S. Li, Zhang, Takemori, Zhou, & Xiong, 2009; Sasaki et al., 2011), and circadian sleep (Jagannath et al., 2013; Sakamoto et al., 2013). Thus, these findings suggest CRTCs have unique functions in a host of biological functions, including metabolism, that are regulated by SIK phosphorylation, but the role of SIK-CRTC1 signaling in sleep is unclear. Ultimately, these downstream targets like HDACs and CRTCs allow SIKs to regulate many biological functions at the level of gene expression.

Work done in mammalian models has identified a multitude of physiological processes and diseases the three SIK isoforms have a role in including depression, tumorigenesis, sleep/circadian rhythms, and metabolism. For instance, SIKs have been shown to act in the hippocampus to regulate depression but the underlying mechanisms are poorly understood. Expression of

SIK2 in the hippocampus appears to be upregulated while CRTC1-CREB signaling is suppressed in mouse models of depression and chronic stress (Jiang et al., 2019). Depending on tumor type, SIKs appear to act both as tumor suppressors and oncogenes (Sun et al., 2020). Disruption of SIK1 and SIK3 increased tumor growth in lung cancer cell models (Murray et al., 2019) while inhibition of SIKs appeared to reduce the severity of myeloid leukemia in mice and ovarian cancer cell models (Tarumoto et al., 2020; J. Zhou et al., 2017). Similarly, disruption of the sleep/wake cycle and circadian rhythmicity varies depending on which SIK is involved. For example, disruption of SIK3 increased sleep need in mice (Funato et al., 2016) while also desynchronizing circadian rhythms (Hayasaka et al., 2021) but mutations in SIK1 cause animals to display behaviors similar to that of jet lag (Jagannath et al., 2013). Likewise in regulating metabolism, inhibition of SIKs induces gluconeogenesis (Patel et al., 2014) while overexpression of SIK1 or SIK2 normalizes blood glucose levels in diabetic mice (Koo et al., 2005).

Together, these findings demonstrate that SIK-HDAC4 and SIK-CRTC1 signaling have essential physiological functions, but their role in sleep and how these pathways are connected to metabolism is poorly understood. As discussed above, SIKs are well-studied and extensively involved in a range of biological processes however, there is still much to be uncovered about their regulation and physiological roles.

4. *C. elegans* as a model to study SIK signaling

C. elegans is a well-established and simple model that is widely used to study an array of biological topics. *C. elegans* is a free-living, transparent, bacterivore that can be found in the soil of temperate environments worldwide (Andersen et al., 2012; Hodgkin & Doniach, 1997; Kiontke et al., 2011). In the lab, *C. elegans* are cultured on agar plates seeded with *Escherichia coli* bacteria as a food source (Brenner, 1974). *C. elegans* are anatomically simple composed of only 959 somatic cells, 302 of which are neurons. Their short life cycle of 3 days consists of four larval stages (L1, L2, L3, L4) and adulthood and have an average lifespan of 2-3 weeks (Byerly, Cassada, & Russell, 1976; Strange, 2006). The worm has two sexes: self-fertilizing hermaphrodites, and males that arise spontaneously. This allows for a high ease of genetic manipulation where males can be easily crossed with hermaphrodite animals to introduce new alleles into strains to produce both self- and cross-progeny. In *C. elegans,* there is one SIK homolog called KIN-29 while there are three SIKs in mammals, and two in *Drosophila* fruit flies (**Figure 2**) (Lanjuin & Sengupta, 2002). Hence, the *C. elegans* lone homolog simplifies genetic manipulations allow easy elimination or restoration of SIK function. KIN-29 is involved in the regulation of many biological functions including gene expression, longevity, metabolism, and sleep, each of which will be explored in the sections below.

5. The effects of SIK signaling and diet on the regulation of chemoreceptor gene expression

Animals rely on external cues and internal signals to appropriately respond to their environments, but what are the mechanism behind their behavioral output? One method that has been hypothesized is that animals can alter their chemosensory behavior by dynamically changing the expression of chemoreceptor genes. For example, the mosquito *Anopheles gambiae* modifies the gene expression of AgOr1, an olfactory receptor that responds to the odor in human sweat to change its host-seeking behavior depending on its feeding state (Hallem, Nicole Fox, Zwiebel, & Carlson, 2004). It was found that AgOr1 expression is high before animals have a blood-feeding but rapidly downregulated post-feeding supporting the hypothesis that animals alter their sensitivity to environmental odor cues to modify their behavior (Fox, Pitts, Robertson, Carlson, & Zwiebel, 2001). Similarly, modification of olfactory behavior by feeding/fasting states has been shown in mammalian models as well in which fasted rats have increased olfactory acuity compared to their satiated counterparts (Aimé et al., 2007) and satiated primates decrease their response to the odors of satiating foods (Critchley & Rolls, 1996). External cues of food availability and internal feeding state signals can also modify behavior in a sex-dependent manner. In *C. elegans*, behavioral prioritization depends on developmental stage, feeding state, and sex (Ryan et al., 2014; Sengupta, Chou, & Bargmann, 1996). The chemoreceptor gene *odr-10* is known to sense the compound diacetyl, a chemical that produces a buttery odor and represents a food signal (Sengupta et al., 1996). Male *C. elegans* prioritize exploration over feeding and will abandon food to instead search for mates, unlike

hermaphrodites (Lipton, Kleemann, Ghosh, Lints, & Emmons, 2004). *odr-10* expression is low when animals are well-fed but high when starved, thus, males will modify their behavior to prioritize food over mate search when starved (Ryan et al., 2014). Additionally, changes in *odr-10* expression between sexes do not diverge until animals reach sexual maturity suggesting *odr-10* regulates behavioral choice in an age-dependent manner as well (Ryan et al., 2014). These findings support the hypothesis that animals may dynamically alter their chemoreceptor gene expression as a mechanism to modify their behavioral responses.

While dynamic changes in the expression of chemoreceptor genes may be, in part, responsible for behavioral modifications, additional modulators of chemoreceptor gene expression are still unknown. Specifically in *C. elegans*, chemoreceptor genes are also known to be regulated by development, sensory activity, and pheromone levels (Nolan, Sarafi-Reinach, Horne, Saffer, & Sengupta, 2002; Peckol, Troemel, & Bargmann, 2001; Tobin et al., 2002). Another regulator of a subset of chemoreceptor genes is the KIN-29/SIK signaling pathway (Lanjuin & Sengupta, 2002; van der Linden et al., 2007). Animals with mutations in *kin-29* are unable to properly sense and process food signals because of altered expression of these chemoreceptor genes in multiple chemosensory neuron types (Lanjuin & Sengupta, 2002). KIN-29 regulates gene expression by inhibiting the repressive effects of downstream target HDA-4 that acts via the transcription factor MEF2 (van der Linden et al., 2007). In the KIN-29 dependent chemoreceptor gene *srh-234,* expression of *srh-234* is repressed

by MEF-2 in the KIN-29 signaling pathway in starved animals (Gruner et al., 2014). Similarly, in *Drosophila,* the inactivation of SIK3 upon starvation results in dephosphorylation of HDAC4 and subsequently reduced activity of the transcription factor FOXO-mediated gene expression (B. Wang et al., 2011).

The presence or absence of food can regulate chemoreceptor genes but there is also evidence displaying the effects of diet on gene expression. It has been shown that long-term consumption of a “Western-style” diet high in saturated fats, sugar, sodium and low in nutrients and fiber has detrimental effects on health, brain function, and behavior (López-Taboada, Gonzalez-Pardo, & Conejo, 2020; Mente, de Koning, Shannon, & Anand, 2009). In humans, participants with a Western-style diet had significantly poorer odor identification ability and fat discrimination as well as altered taste and flavor perception (Stevenson et al., 2016). Similarly, mice fed high fat or Western-style (high fat and sugar) diets had reduced ability to detect, remember, and learn new odors, and these olfactory learning and memory deficits were not associated with diet-induced obesity but of the high sugar diet itself (Lietzau, Nyström, Wang, Darsalia, & Patrone, 2020). Interestingly, SIK3 expression is induced in mice fed a western style diet high in fat, sugar, and cholesterol (Uebi et al., 2012) suggesting a link between SIKs and diet-dependent changes in behavior. As shown above, the modulatory effects of diet on chemosensory behavior and gene expression are prominent but the mechanisms are not entirely clear.

6. The effects of SIK and HDAC4 signaling in lifespan regulation

Aging is a universal but complex physiological process mediated by many biological and genetic pathways. This phenomenon is characterized by irreversible and progressive decline physiological functions and cellular senescence (Z. Li et al., 2021; López-Otín, Blasco, Partridge, Serrano, & Kroemer, 2013). As organisms age, their DNA repair mechanisms become defective causing a cascade of detrimental effects on gene expression and transcription pathways and ultimately leading to cellular dysfunction (Z. Li et al., 2021). Aging, through this decline of function and accumulation of genetic damage, can lead to age-related diseases including cardiovascular disease (Donato, Machin, & Lesniewski, 2018), metabolic disorders (Arriola Apelo & Lamming, 2016), mobility disorders (Grote, Reinhardt, Zhang, & Wang, 2019), cancer (Smith, Smith, Hurria, Hortobagyi, & Buchholz, 2009), and is the most common risk factor of neurodegenerative disease (Kubben & Misteli, 2017; Z. Li et al., 2021; Trevisan, Cristina-Pereira, Silva-Amaral, & Aversi-Ferreira, 2019). However, the underlying molecular mechanisms of aging remain uncertain.

SIKs appear to be involved in lifespan, for instance mutants of the SIK homolog KIN-29 are observed to have extended lifespan phenotypes (Lanjuin & Sengupta, 2002), but the exactly regulatory mechanism are unknown. Downstream substrates of SIKs, like HDAC4 (Di Giorgio et al., 2021; X. Han et al., 2016) have been shown to be involved in aging but it is unknown whether

these substrates have a role in the KIN-29 pathway to regulate lifespan. HDACs control the balance of histone acetylation and deacetylation (Ropero & Esteller, 2007). These HDACs can regulate longevity and other cellular functions by modifying histones and chromatin structures as well as non-histone proteins like transcription factors and other regulatory proteins (Chen et al., 2015; Glozak, Sengupta, Zhang, & Seto, 2005). The class II histone deacetylase, HDAC4, is known to act as a transcriptional repressor that shuttles between the cytoplasm and the nucleus depending on phosphorylation state by SIK (Haberland et al., 2009; Miska et al., 1999; Verdin, Dequiedt, & Kasler, 2003). This class of HDACs bind to and inhibit the MEF-2 family of transcription factors and play a role in muscle-specific gene activation and differentiation (McKinsey, Zhang, & Olson, 2001; Miska et al., 1999) but the role of each HDAC and its downstream targets in the regulation of lifespan is uncertain.

Genetic lifespan studies have contributed the most to our understanding of the role of SIKs and HDACs in longevity. In mammals, genetic knock-down of HDAC4 increases lifespan in mouse models of Huntington's disease, a neurodegenerative disorder known to shorten life expectancy following onset of symptoms (Solberg, Filkuková, Frich, & Feragen, 2018). Interestingly, in *Drosophila* epilepsy models, the SIK3-HDAC4 pathway is inactive, and animals display neuronal hyperactivity and shortened lifespan suggesting SIK3-HDAC4 signaling is involved in lifespan regulation (H. Li, Lones, & DiAntonio, 2021). In human fibroblast cells, HDAC4 knockdown results in premature cellular aging while overexpression of HDAC4 was reported to delay cellular senescence (X.

Han et al., 2016) proposing HDAC4 expression as a mechanism to regulate aging. This SIK-HDAC mediated regulation of lifespan is also observed in *C. elegans* and will be explored in work found in **Chapter 3**.

Understanding the role of SIK and HDACs in lifespan sheds light on the underlying mechanisms of the gene regulation of aging. Identifying additional genes altered by HDAC and its inhibitors will provide new areas of investigation and potential targets of drug therapies for age-related diseases.

7. The effects of SIK signaling on sleep and metabolism

Sleep and metabolism are intricately connected and the increase in metabolic disorders coincides with the epidemic of chronic short or fragmented sleep. During normal sleep, the rate of human metabolism is reduced by approximately 15%, reaching a minimum by morning (Brebbia & Altshuler, 1965; Goldberg, Prentice, Davies, & Murgatroyd, 1988). Correlative studies have shown that individuals that slept for 6 hours or less per night or more than 9 hours had higher body mass indexes (BMIs) the latter likely due to comorbidities like obstructive sleep apnea (Cappuccio et al., 2008; Carter & Watenpaugh, 2008; Kohatsu et al., 2006). The lowest BMIs were associated with individuals who slept 7-8 hours per night (Taheri, Lin, Austin, Young, & Mignot, 2004). While these studies she light on the correlation between sleep and metabolism, little is known about the causality and neural and molecular mechanisms that underlie

this interaction (Kohatsu et al., 2006; Sharma & Kavuru, 2010; Taheri et al., 2004; Zhao, Zhao, & Veasey, 2017).

7.1. *C. elegans* as a model to study sleep and metabolism

Many genetic factors that control fat storage and energy metabolism in mammals are conserved in *C. elegans* (Ashrafi et al., 2003; Lai, Chou, Ch'ang, Liu, & Lin, 2000; Soukas, Kane, Carr, Melo, & Ruvkun, 2009). There are also a variety of tools available to asses metabolic activity in worms including measurement of fat stores and oxygen consumption making it a powerful model to uncover the molecular pathways affected in metabolic disorders (O'Rourke, Soukas, Carr, & Ruvkun, 2009; Soukas et al., 2009; Suda, Shouyama, Yasuda, & Ishii, 2005). Energy in *C. elegans* is stored as glycogen and triglycerides like in mammals and can be mobilized during periods of high energetic demand in response to stress and to promote growth and development (Watts & Ristow, 2017). Additionally, the nervous system of the worm acts as a central command and control center involved in nutrient sensing and signaling to regulate metabolism resembling the mammalian hypothalamus (Hashmi et al., 2013; Hussey et al., 2017).

C. elegans satisfies all behavioral hallmarks of sleep including reversible quiescent behavior, increased arousal threshold, stereotypical posture, a homeostatic response to sleep deprivation, as well as sharing conserved genetic

signaling pathways like EGF and SIK (**Table 1**) (Grubbs, Lopes, van der Linden, & Raizen, 2020; Iwanir et al., 2013; Raizen et al., 2008; Trojanowski & Raizen, 2016). There are two sleep states exhibited by *C. elegans* known as developmentally timed sleep (DTS) and stress-induced sleep (SIS) as well as starvation and satiety-associated sleep (Davis & Raizen, 2017; Hill, Mansfield, Lopez, Raizen, & Van Buskirk, 2014; Raizen et al., 2008; You, Kim, Raizen, & Avery, 2008). DTS or lethargus occurs at the end of each of the four larval stages of the worm lasting about 2-3 hours during which animals shed their cuticle as they develop into adulthood (Raizen et al., 2008). This sleep state is characterized with animals presenting behavioral quiescence, a stereotypical posture, an increased arousal threshold, cessation of feeding, and a homeostatic response to sleep deprivation (Iwanir et al., 2013; Raizen et al., 2008). DTS is analogous to circadian sleep in *Drosophila* and humans and is similarly regulated by homologs of circadian clock genes (Davis & Raizen, 2017; Dunlap, 1999). The second sleep state SIS occurs following environmental exposure to cellular stress or injury such as ultraviolet (UV) or heat-shock (Hill et al., 2014; Trojanowski & Raizen, 2016). The duration of SIS depends on the severity of the stressor and appears to be a protective state induced by cellular stress to mitigate disrupted cellular homeostasis (Hill et al., 2014; Jones & Candido, 1999). Both sleep states in *C. elegans* can be easily measured using high-throughput systems to measure total minutes or fraction of sleep over several hours (Churgin & Fang-Yen, 2015; Churgin et al., 2019).

7.2. The role of SIKs in metabolism and sleep

SIKs are well known conserved regulators of metabolism (Sakamoto et al., 2018) and have recently been shown to be involved in the regulation of sleep (Funato et al., 2016; Grubbs, Lopes, et al., 2020). The role of SIKs in metabolism is generally recognized and accepted. SIKs, like AMPKs, are activated by the liver kinase B1 (LKB1) and target tissues involved in metabolism such as the liver, adipose tissue, and muscles to promote metabolic homeostasis (Nixon et al., 2016; Patel et al., 2014; Säll et al., 2017). Several studies have demonstrated the key role SIKs play in controlling metabolism in several signaling pathways in these various cells and tissues. For instance, SIK3 knockout mice have altered lipid and glucose metabolism and display a malnourished phenotype despite being fed a high-fat diet (Uebi et al., 2012). Similarly, knockout mice of SIK2 had increased blood glucose levels related to compromised glucose and insulin tolerance in adipocytes (Jinyoung Park et al., 2014) while SIK1 knockout mice fed a high fat diet show increased insulin sensitivity (Nixon et al., 2016). Together, these findings demonstrate that all three SIKs play an important role in metabolic regulation but how they link metabolism to sleep?

A forward genetic screen found that SIK3 gain-of-function mice display increased sleep and have a phosphoprotein profile reminiscent of sleep-deprived animals suggesting SIK3 promotes sleep (Funato et al., 2016; Z. Wang et al., 2018). Recently, a short isoform of SIK3 has also been identified to critically contribute to increase of sleep quantity and intensity in mice (Xu et al., 2022).

Moreover, SIK3 deficient mice display abnormal patterns of circadian rhythms including lengthened period, impaired light-dark entrainment, and destabilized molecular rhythms (Hayasaka et al., 2021). Comparably, SIK3 mutants of *Drosophila* and KIN-29 mutants of *C. elegans* also exhibit reduced sleep phenotypes again demonstrating this role for SIK3/KIN-29 in sleep regulation is conserved (Funato et al., 2016; Grubbs, Lopes, et al., 2020). Like in metabolism, SIK1 and SIK2 have recently been shown to be involved in the homeostatic regulation of sleep. Animals with mutations in the PKA phosphorylation site of SIK1 and SIK2 mutants display an increased need for sleep (M. Park et al., 2020) therefore revealing that the well-conserved PKA phosphorylation site of SIKs (**Figure 2B**) appears to be involved in the regulation of sleep need. While these other two SIK isoforms have been shown to be involved in sleep, their effects appear to be small compared to the role of SIK3 in sleep.

7.3. The role of growth factors in sleep

The bulk of sleep research focuses on understanding natural, healthy sleep that occurs in a 24 hour-circadian rhythmicity. In addition to natural sleep, sleepiness or fatigue occurs during illness or infection (Davis & Raizen, 2017). In response to cellular injury, small proteins called cytokines are secreted by immune cells and induce somnogenic effects (Eriksson, Nobel, Winblad, & Schultzberg, 2000). One group of cytokines from the epidermal growth factor (EGF) family are known to be involved in development, expressed in the brain,

and similarly released in response to cellular injury and stress (Fischer, Hart, Gschwind, Prenzel, & Ullrich, 2004; Jin et al., 2002). Studies in mammalian sleep have shown that EGF increases sleep (Kramer et al., 2001; Kushikata, Fang, Chen, Wang, & Krueger, 1998) but the mechanisms and signaling targets are still unidentified.

Epidermal growth factor (EGF) found in the brain is a growth factor that helps coordinate cell growth, proliferation, differentiation, and maintenance through DNA synthesis and cell division (Kushikata et al., 1998). EGF also stimulates the production of several sleep promoting substances suggesting a role in sleep regulation (Kapás, Fang, & Krueger, 1994; Kapás, Shibata, Kimura, & Krueger, 1994). Work in mammals have shown that administration of exogenous EGF increases sleep (Kramer et al., 2001; Kushikata et al., 1998) but the underlying mechanism of growth factors on sleep is still poorly understood. Similarly, the single EGF homolog in *C. elegans,* LIN-3, has similarly been shown to act downstream of KIN-29/SIK to regulate quiescence (Hill et al., 2014; Konietzka et al., 2020a; Van Buskirk & Sternberg, 2007).

Another growth factor transforming growth factor beta (TGF-β), is a widely expressed protein that plays a key role in multiple processes ranging from immune response, cell growth, cancer, and sleep (Colak & Ten Dijke, 2017; Gast et al., 2012; Goetting, Soto, & Van Buskirk, 2018; Itoh, Watabe, & Miyazono, 2014; You et al., 2008). Recent work shows that TGF-β signaling is important for normal circadian clock function in zebrafish (Sloin et al., 2018) and mammals (Kon et al., 2008) suggesting the role of TGF-β signaling in sleep and circadian

rhythms is highly conserved. Moreover, the *C. elegans* TGF-β homolog, DAF-7 has been shown to be involved in the regulation of satiety quiescence. After fasting and refeeding, *daf-7* mutants display reduced quiescence compared to wild-type animals (You et al., 2008). These findings suggest this food/satiety-dependent quiescence is mediated by DAF-7/TGF-β signaling.

8. Concluding remarks

Understanding animal-environment interactions is a complex topic with many prospective areas of research to explore. The KIN-29/SIK signaling pathway acting in a subset of sensory neurons is where signals from external stimuli and internal states converge and may be a mechanism through which animals to optimally fine-tune their behavior. SIKs have been well established as regulators of internal state (Gruner et al., 2014) and are now emerging as regulators of sleep and metabolism (Funato et al., 2016; Grubbs, Lopes, et al., 2020) but, the underlying mechanisms remain unknown. *C. elegans* is a powerful, simple model to understand the genetic and neural basis of these behaviors.

OVERVIEW

My dissertation work aims to uncover the molecular mechanisms that underlie how animals integrate external and internal signals to respond to their environment. I postulate that these signals converge in a subset of sensory neurons where an AMPK-subfamily member, SIK3, acts to control gene expression, development, and behavior. To address my central hypothesis, using the nematode *C. elegans* I have studied its sole SIK homolog, KIN-29, in its role of environmental and internal state signals and investigated its candidate downstream targets in the regulation of chemoreceptor gene expression and sleep in the following chapters.

Chapter 2: The widespread expression of SIK and its many downstream targets, leads us to hypothesize that SIK acts in a subset of sensory neurons to regulate multiple different pathways. In this chapter, I describe the role of SIK in the regulation of chemoreceptor gene expression. We previously identified a KIN-29/SIK dependent chemoreceptor, *srh-234*, expressed in the ADL sensory neuron of *C. elegans* that is regulated by feeding state and internal starvation signals via the MEF-2 transcription factor. We now show that *srh-234* expression is also regulated by the micronutrient vitamin B12 obtained through diet which appears to be distinct from the starvation response. Animals fed the vitamin B12 producing bacteria *Comamonas aquatica* have reduced *srh-234* expression compared to animals fed a standard B12-deficient *E. coli* diet. Similarly, mutant *Comamonas aq.* bacteria that are no longer able to produce vitamin B12 do not

downregulate *srh-234* expression. This suggests *srh-234* is regulated by *Comamonas*-diet-derived vitamin B12. Supplementation of metabolically active vitamin B12 to the *E. coli* diet also repressed *srh-234* expression indicating *E. coli* may function as a vehicle for vitamin B12 uptake. These vitamin B12 repressive effects on *srh-234* expression are suppressed by excessive levels of propionate as well as perturbations in the genetic propionate breakdown pathways. Lastly, we found that the MEF-2 transcription factor and a yet unknown factor (X) act together to regulate *srh-234* expression allowing animals to fine-tune their chemosensory responses to their environment at the transcriptional level.

Chapter 3: The phenomenon of aging and cellular senescence is an inevitable and universal process. However, the regulation of aging and longevity is not fully understood. This chapter describes the role of KIN-29/SIK as a regulator of lifespan. We show that the previously identified extended lifespan of *kin-29* mutant animals is dependent on the *C. elegans* HDAC4 homolog HDA-4. Through survival analysis, we now show that *hda-4* mutant animals are short lived. Double mutants of *kin-29* and *hda-4* display longevity phenotypes similar to *hda-4* single mutants. These results suggest that KIN-29 may regulate lifespan via the downstream action of HDA-4.

Chapter 4: Sleep and metabolism are shown to be highly interconnected. SIK and its downstream targets are demonstrated in the regulation of both these independent pathways. This supports my central hypothesis of the role of SIK in regulating multiple pathways. The third chapter in my dissertation explores the

role of downstream targets of KIN-29/SIK in the metabolic regulation of sleep. KIN-29/SIK is a key regulator of sleep and metabolism that acts in glutamatergic neurons and has many downstream phosphorylation targets including the class IIa histone deacetylase HDA-4 and the CREB-regulated transcription cofactor CRTC-1. Here we show that CRTC-1 regulates stress-induced sleep. Similar to *kin-29*, *crtc-1* mutant animals have reduced SIS and this phenotype can be rescued by overexpressing *crtc-1* under the control of its own promoter. *crtc-1; kin-29* double mutants display a more severe defective movement quiescence phenotype than the single mutants suggesting *crtc-1* and *kin-29* may act in parallel in glutamatergic neurons. Mutants of known interactor CRTC-1, the CREB homolog *crh-1*, have reduced SIS. We show that both *crtc-1* and *crh-1* act in the KIN-29 signaling pathway upstream of EGF receptor signaling and sleep promoting neurons ALA/RIS. Similar to *kin-29*, loss-of-function mutations in *hda-4* corrects the reduced SIS phenotype of *crtc-1* mutant animals. While *crtc-1* mutants are defective in movement quiescence they are not defective in feeding quiescence. Interestingly, this sleep phenotype is similar to mutants of *daf-7*/TGF-β that are involved in the food-dependent plasticity of SIS where quiescence is exhibited following a fast. Moreover, *crtc-1* and *crh-1* strongly reduce *daf-7* expression in the ASI neuron. These data suggest that CRTC-1 may be acting in both the KIN-29/SIK and food-dependent DAF-7/TGF-β signaling pathways as a new regulator of SIS.

CHAPTER II: Diet regulates chemosensory receptor gene expression via the MEF2 transcription factor in *Caenorhabditis elegans*

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ABSTRACT

Dynamic changes in chemoreceptor gene expression levels in sensory neurons is one strategy that an animal can use to modify their responses to dietary changes. However, the mechanisms underlying diet-dependent modulation of chemosensory gene expression are unclear. Here, we show that the expression of the *srh-234* chemoreceptor gene localized in a single ADL sensory neuron type of *C. elegans* is downregulated when animals are fed a *Comamonas aquatica* bacterial diet, but not on an *Escherichia coli* diet. Remarkably, this diet-modulated effect on *srh-234* expression is dependent on the micronutrient vitamin B12 endogenously produced by *Comamonas aq.* bacteria. Excess propionate and genetic perturbations in the canonical and shunt

propionate breakdown pathways are able to override the repressive effects of vitamin B12 on *srh-234* expression. The vitamin B12-mediated regulation of *srh-234* expression levels in ADL requires the MEF-2 transcription factor, providing a potential mechanism by which dietary vitamin B12 may transcriptionally tune individual chemoreceptor genes in a single sensory neuron type, which in turn may change animal responses to biologically relevant chemicals in their diet.

1. INTRODUCTION

Animals receive dietary inputs from their environment and their internal metabolic state, which allows them to modify their chemosensory response properties and behavioral outcomes (Sengupta, 2012). One strategy that animals can use to trigger long-term changes in behavioral outcomes is by dynamically changing the expression of individual chemoreceptor genes localized in different chemosensory neuron types. These dynamic transcriptional changes in chemoreceptor gene expression in response to food and internal feeding state is observed in mosquitoes and play pivotal roles in their ability to seek food and reproduce (Fox et al., 2001; Hallem et al., 2004; Khan et al., 2021; Rinker et al., 2013; Ryan et al., 2014; Taparia, Ignell, & Hill, 2017), but the mechanisms controlling this plasticity in chemoreceptor gene expression are unclear.

The nematode *Caenorhabditis elegans* is an excellent model organism to study interactions between an animal and its dietary sources (Yilmaz & Walhout, 2014; J. Zhang, Holdorf, & Walhout, 2017). *C. elegans* is a bacterivore, making it

easy to expose these animals to different bacterial strains to study their effects on organismal health and physiology. Bacterially derived factors affect *C. elegans* in various ways; for instance, pathogenic factors are sensed by chemosensory neurons and trigger avoidance behaviors (Meisel, Panda, Mahanti, Schroeder, & Kim, 2014; Pradel et al., 2007), while other bacterially derived factors are innocuous and contribute to physiology and development (Coolon, Jones, Todd, Carr, & Herman, 2009; Gracida & Eckmann, 2013). Recent work demonstrated that vitamin B12 obtained by *C. elegans* through its bacterial diet is an important nutritional factor in developmental growth and physiology of *C. elegans* (MacNeil, Watson, Arda, Zhu, & Walhout, 2013). The vitamin B12 status of *C. elegans* can be easily assessed with help of the *acdh-1p::GFP* reporter, which is expressed in response to propionate accumulation resulting from B12 deficiency (Watson, MacNeil, Arda, Zhu, & Walhout, 2013; Watson et al., 2014b; Watson et al., 2016). When fed a vitamin B12-deficient *Escherichia coli* OP50 diet, *acdh-1* is highly expressed in animals, whereas *acdh-1* is lowly expressed when grown on the vitamin B12-producing *Comamonas aq.* DA1877 diet. The effects of these bacterial diets on *acdh-1* promoter activity have led to important insights into the vitamin B12-dependent and independent propionate breakdown pathways.

Caenorhabditis elegans is also an ideal organism to study the plasticity in expression levels of individual chemosensory receptor genes in response to external and internal signals (Gruner & van der Linden, 2015; Vidal et al., 2018). Our prior study showed that the expression levels of the *srh-234* chemoreceptor

gene in the ADL sensory neuron type is regulated by starvation. This starvation-mediated modulation of *srh-234* expression levels is dependent on sensory inputs into ADL neurons perceiving food presence, and circuit inputs from RMG interneurons that are electrically connected to ADL perceiving internal state of starvation signals (Gruner et al., 2014). Circuit inputs from RMG into ADL regulating *srh-234* required the NPR-1 neuropeptide receptor acting in RMG, as well as insulin signals from other tissues acting on the DAF-2 insulin receptor in ADL (Gruner et al., 2014). In addition, starvation-mediated regulation of *srh-234* expression levels in ADL is regulated by both cell- and non-cell-autonomous transcriptional mechanisms involving basic helix-loop-helix (bHLH) factors, including HLH-30 and MXL-3 acting in the intestine, and HLH-2/3 acting together with the MEF-2 factor in ADL neurons (Gruner et al., 2016). Together, these findings demonstrated that expression of the *srh-234* chemoreceptor gene in a single ADL sensory neuron type of *C. elegans* is regulated by multiple transcriptional modules, and revealed a neuron-to-intestine connection involving insulin signals in the modulation of chemoreceptor genes as a function of the *C. elegans* feeding state (Gruner & van der Linden, 2015).

In this study, we discovered that feeding *C. elegans* vitamin B12-producing *Comamonas aq.* bacteria regulates the expression levels of the *srh-234* chemoreceptor gene in ADL neurons. We show that *srh-234* gene expression is repressed in ADL when animals are fed a high vitamin B12 diet of *Comamonas aq.* DA1877 bacteria relative to a low vitamin B12 diet of *E. coli* OP50 bacteria. This dietary effect of vitamin B12 on *srh-234* in ADL appears

to be distinct from the starvation response we reported previously (Gruner et al., 2014). Mutant bacteria of *Comamonas aq.* deficient in vitamin B12 production, indicated that *Comamonas*-supplied vitamin B12 regulates *srh-234* expression levels in ADL. Similar to feeding *C. elegans* a *Comamonas aq.* diet, supplementing the *E. coli* diet with exogenous vitamin B12 also represses *srh-234* expression in ADL neurons, in which *E. coli* may function as a vehicle for vitamin B12 uptake by *C. elegans*. The repressing effects of vitamin B12 on *srh-234* expression in ADL neurons can be suppressed by excess propionate supplementation and genetic perturbations in the canonical and shunt propionate breakdown pathways. Lastly, we show that the MEF-2 transcription factor and its binding site in the *srh-234 cis*-regulatory sequence are necessary for the vitamin B12-mediated repression of *srh-234* expression in ADL. Together, these findings reveal that bacterially derived vitamin B12 turn individual chemoreceptor genes on and off at the level of transcription in sensory neurons that may inform our understanding of how animals fine-tune their chemosensory responses to biologically relevant chemicals in their diet.

2. RESULTS

The *Comamonas aq.* DA1877 diet represses *srh-234* expression in ADL neurons

To study how bacterial diet regulates chemoreceptor gene expression levels in *C. elegans*, we used the candidate *srh-234* chemoreceptor gene

specifically expressed in a single sensory neuron type, ADL. We previously found that GFP expression driven by only 165 bp *cis*-regulatory sequence of *srh-234* (referred here as *srh-234p::GFP*) is rapidly (<1 h) downregulated in starved animals (Gruner et al., 2014). While testing the *srh-234p::GFP* reporter in different bacterial diets, we observed that animals fed a *Comamonas aq.* DA1877 diet repress *srh-234* expression in ADL neurons similar to that observed in starved animals (Gruner et al., 2014); that is *srh-234p::GFP* expression levels in adult animals is strongly decreased when fed a *Comamonas aq.* DA1877 diet compared to a *E. coli* OP50 diet (**Figure 3A**). This *Comamonas*-mediated repression of *srh-234* expression levels is rapid as adult animals raised on *E. coli* OP50 and then transferred to a DA1877 diet decrease *srh-234p::GFP* expression in ADL by 50% about 2 h after the transfer (**Figure 4A**). Animals fed other *E. coli* diets such as the K12/B-type hybrid HB101 strain, and the K12-type HT115 strain commonly used in *C. elegans* research showed a *srh-234* expression phenotype intermediate to that of *E. coli* OP50 and *Comamonas aq.* DA1877 diets (**Figure 4B**).

The dietary effect of *Comamonas aq.* DA1877 on *srh-234* expression appears to be distinct from the starvation response, because mixing the *E. coli* OP50 diet with *Comamonas aq.* DA1877 diet 1:1 resulted in animals in which *srh-234* expression levels remained strongly decreased similar to starvation (**Figure 3B**). Moreover, smaller concentrations of *Comamonas aq.* DA1877 by diluting it in *E. coli* OP50 (i.e., 9:1 and 99:1 OP50/DA1877) was sufficient to strongly repress *srh-234* expression. Others have reported

that *Comamonas aq.* DA1877 bacteria are not a nutrient-poor diet for *C. elegans* (MacNeil et al., 2013; Shtonda & Avery, 2006), suggesting that *Comamonas aq.* DA1877 may generate a bacterial signal that regulates *srh-234* expression levels. This dietary effect of *Comamonas* on *srh-234* may be specific since expression of another ADL-specific *sre-1* chemoreceptor is not affected (**Figure 4C**). Since we previously showed that lack of functional ADL cilia can dramatically decrease *srh-234p::GFP* expression levels (Gruner et al., 2014), it is possible that the *Comamonas* diet affects the integrity of ADL neurons resulting in the reduced *srh-234* expression. However, we found that wild-type animals fed a *Comamonas aq.* DA1877 diet show normal ADL morphology determined by *sre-1p::GFP* expression (**Figure 4D** and **4E**) and wild-type dye-filling (**Figure 4F**) ruling out cilia defects of ADL neurons fed on the *Comamonas aq.* DA1877 diet. Together, these results suggest that in addition to starvation, a dilutable bacterial metabolite produced by *Comamonas aq.* DA1877 bacteria represses *srh-234* expression levels in ADL neurons.

Vitamin B12 produced by *Comamonas aq.* represses *srh-234* expression

The strain *Comamonas aq.* DA1877 produces the dilutable metabolite vitamin B12, while the *E. coli* OP50 strain is not able to synthesize vitamin B12 (Watson et al., 2014b). To test the hypothesis that vitamin B12 represses *srh-234* expression levels in ADL neurons, we examined *C. elegans* animals fed a *E. coli*

OP50 diet supplemented with two biologically active and interconvertible forms of vitamin B12, adenosylcobalamin (Ado-Cbl) and methylcobalamin (Me-Cbl). We found that animals fed an *E. coli* OP50 diet supplemented with either 64 nM vitamin B12 (Ado-Cbl or Me-Cbl) repress *srh-234p::GFP* expression in ADL neurons (**Figure 5A**). Moreover, supplementing *E. coli* OP50 with increasing concentrations (nM doses) of vitamin B12 (Me-Cbl) resulted in a dose-dependent decrease in *srh-234p::GFP* expression (**Figure 6A**), which fits with our observation that diluting *Comamonas aq.* DA1877 into the *E. coli* OP50 diet is sufficient to repress *srh-234* expression (**Figure 3B**). As a control, we found similar dose-dependent repressive effects of vitamin B12 (Me-Cbl) using the *acdh-1p::GFP* dietary reporter expressed primarily in the intestine (MacNeil et al., 2013; Watson et al., 2014b) when fed either the vitamin B12-producing *Comamonas aq.* DA1877 or the *E. coli* OP50 diet supplemented with vitamin B12 (Me-Cbl) (**Figure 6B**). These results are also consistent with the observed decrease in *srh-234* expression when animals were fed on *E. coli* HT115 and *E. coli* HB101 diets (**Figure 2B**), which are better sources of vitamin B12 levels than *E. coli* OP50 (Revtovich, Lee, & Kirienko, 2019). Thus, vitamin B12 supplementation to the *E. coli* OP50 diet can repress *srh-234* expression in ADL neurons.

To further test whether *Comamonas*-supplied vitamin B12 regulates *srh-234* expression in ADL, we took advantage of two mutant strains of *Comamonas aq.* bacteria (Δ*cbiA* and Δ*cbiB*) that are deficient in vitamin B12 production (Watson et al., 2014b). We found that feeding animals Δ*cbiA* and Δ*cbiB* bacterial

mutants fail to repress *srh-234p::GFP* expression compared to DA1877-fed animals (**Figure 5C**). As expected, Δ*cbiA* and Δ*cbiB* bacterial mutants also fail to repress *acdh-1* expression as determined by the *acdh-1p::GFP* reporter (**Figure 6D**). Together, these findings suggest that vitamin B12 synthesized by *Comamonas aq.* DA1877 bacteria repress *srh-234* expression in ADL.

Escherichia coli may act as a conduit for vitamin B12 uptake by *C. elegans,* to in turn regulate *srh-234* expression

Since *E. coli* OP50 bacteria supplemented with exogenous vitamin B12 can repress *srh-234* expression, we reasoned that *E. coli* may function as a vehicle for the provision and uptake of vitamin B12 by *C. elegans*, to in turn regulate *srh-234* expression in ADL neurons. Alternatively, it remains possible that the availability of vitamin B12 in these supplementation experiments can also be directly perceived by ADL and regulate *srh-234*. To distinguish between these possibilities, we first tested whether vitamin B12 can be perceived as a volatile olfactory cue by ADL neurons to regulate *srh-234*. We exposed animals to live *Comamonas aq.* DA1877 which they cannot eat or touch, while feeding live *E. coli* OP50, and vice versa (**Figure 7A**). In addition, we exposed animals expressing *srh-234p::GFP* to plates seeded with live *E. coli* OP50 that were covered with petri-dish lids containing NGM agar squares soaked with 1 mM of vitamin B12 (Me-Cbl) placed above the animals (**Figure 8A**). In both olfactory

assays, we found that *srh-234* expression in ADL was not significantly altered when exposed to volatile cues of *Comamonas aq.* bacteria or the addition of exogenous vitamin B12. Thus, vitamin B12 may not act as a volatile chemical cue to repress *srh-234* expression, but we cannot exclude the possibility that vitamin B12 may still be directly sensed by ADL neurons.

We next tested whether *E. coli* bacteria could act as a vehicle for vitamin B12 uptake by *C. elegans* in vitamin B12-supplementation experiments. The *tonB* transporter in *E. coli* has been shown to be vital for vitamin B12 uptake from its extracellular environment (Bassford, Bradbeer, Kadner, & Schnaitman, 1976; Kadner, 1990). Recent work showed that *C. elegans* animals fed an *E. coli* BW25113 diet in which the *tonB* transporter gene is deleted (Δ*tonB*) can block, at least in part, the repression of the *acdh-1p::GFP* reporter when the growth media is supplemented with vitamin B12 (Revtovich et al., 2019). Based on these observations, we hypothesized that the effects of vitamin B12 on repressing *srh-234* expression would also be dependent on the *E. coli* TonB pathway. We found that supplementing the *E. coli* BW25113 diet with vitamin B12 (Ado-Cbl or Me-Cbl) repressed *srh-234p::GFP* expression similar to that observed for *E. coli* OP50-fed animals supplemented with vitamin B12 (**Figure 7B**).
When *tonB* was deleted (Δ*tonB*), we found a small, but statistically significant ($P < 0.05$) block in the repression of *srh-234* by Ado-Cbl but less so with Me-Cbl (**Figure 7B**). Thus, *E. coli* bacteria via the TonB transporter may be an important determinant for the vitamin B12 uptake by *C. elegans* to in turn regulate *srh-234* expression. However, because Δ*tonB* did not completely block the effects of

vitamin B12 on repressing *srh-234*, it is likely that there are alternative TonB independent routes to obtain vitamin B12 from the media.

To further validate that *E. coli* bacteria acts as a vehicle for vitamin B12 uptake by *C. elegans*, we asked whether heat-killed *E. coli* bacteria supplemented with vitamin B12 could repress *srh-234* expression. Growing worms on heat-killed *E. coli* bacteria is challenging, because heat destroys some of the nutrients needed for worms to develop and renders the bacteria less edible, resulting in larvae that arrest their development (Qi, Kniazeva, & Han, 2017). We therefore exposed animals to live *E. coli* OP50 until adulthood, and then transferred young adults to plates seeded with *E. coli* OP50 killed by high heat (>75 °C) with and without vitamin B12 (Me-Cbl). Adults fed heat-killed *E. coli* OP50 without vitamin B12 (HK-OP50) grew slowly as expected and resulted in animals with significantly reduced *srh-234p::GFP* expression 48 h after their transfer (**Figure 8B**), mimicking the starvation-induced reduction in *srh-234* expression we previously observed (Gruner et al., 2014). However, when we fed adults heat-killed *E. coli* OP50 bacteria with vitamin B12 (HK-OP50+B12), the vitamin B12 supplementation had a beneficial effect as worms grew faster consistent with previous observations (Qi et al., 2017), but *srh-234p::GFP* expression was less repressed by vitamin B12 compared to live *E. coli* OP50 with vitamin B12 (OP50+B12) (**Figure 8B**). We found similar results for the *acdh-1::GFP* dietary reporter (**Figure 8B**).

Thus, consistent with our TonB findings, *E. coli* bacteria in our vitamin B12-supplementation experiments may merely act as a vehicle for vitamin B12

uptake by *C. elegans*, which in turn can repress the expression of *srh-234* in ADL neurons.

Propionate overrides the repressing effects of vitamin B12 on *srh-234* expression

In the presence of vitamin B12 when fed a *Comamonas aq.* DA1877 diet, *C. elegans* preferably uses the canonical vitamin B12-dependent propionate breakdown pathway (**Figure 9A**). However, in low dietary vitamin B12 conditions when fed an *E. coli* OP50 diet, *C. elegans* mainly utilizes and transcriptionally activates its alternative vitamin B12-independent propionate shunt pathway to breakdown toxic propionate buildup (Watson et al., 2013; Watson et al., 2014b; Watson et al., 2016) (**Figure 9A**). The balance between vitamin B12 and propionyl-CoA levels involved in this propionate breakdown controls promoter activity of the *acdh-1* gene; that is, excess propionate is able to override the repressing effects of vitamin B12 on *acdh-1* expression (Watson et al., 2016). Similarly, we found that animals fed an *E. coli* OP50 diet supplemented with both vitamin B12 (Me-Cbl, 64 nM) and excess propionate (40 mM) can override the repressing effects of vitamin B12 on *srh-234p::GFP* expression (**Figure 9B**).

Genetic perturbation of the canonical and propionate shunt breakdown pathway can also lead to propionate accumulation, such that loss of both *pcca-1* and *acdh-1* function results in synthetic lethality likely due to toxic effects of propionate buildup (Watson et al., 2013; Watson et al., 2014b; Watson et al.,

2016). We therefore tested whether propionate buildup due to genetic perturbations in these propionate breakdown pathways also lead to changes in *srh-234* promoter activity. We found that expression of *srh-234p::GFP* in mutants of *mmcm-1* encoding the methylmalonyl-CoA mutase that requires vitamin B12 as a co-factor is not significantly different in animals fed *E. coli* OP50 with or without exogenous vitamin B12 (Me-Cbl) (**Figure 9C**). Thus, consistent with excess propionate overriding the effects of vitamin B12 on *srh-234* expression, *mmcm-1* mutations also block the repressive effects of vitamin B12. Similarly, mutations in *pcca-1* and *pccb-1* genes (**Figure 10A-B**) or in propionate shunt pathway genes, *acdh-1* and *hphd-1* (**Figure 9D**, **10C**) can also, at least in part, block the repressing effects of vitamin B12 on *srh-234* when fed on *Comamonas aq.* DA1877. Interestingly, *srh-234p::GFP* expression is slightly increased in mutants of *mmcm-1*, *pccb-1* and *pcca-1* compared to wild-type animals when fed on *E. coli* OP50, which could be explained by a further accumulation of propionate in these conditions. Mutations in the methionine/SAM cycle gene, *metr-1*, did not show significant differences in *srh-234p::GFP* expression compared to wild-type animals when fed on *Comamonas aq.* DA1877 (**Figure 9E**). All mutants tested showed a normal dye-filling of ADL neurons (**Figure 10D**). These findings suggest that mainly genetic perturbations in the canonical and propionate shunt breakdown pathways can alter *srh-234* expression levels in ADL.

The nuclear hormone receptor, *nhr-68,* is involved in activating shunt gene expression in the *C. elegans* intestine in response to excessive propionate levels,

and mutations in *nhr-68* are proposed to mitigate propionate toxicity (Bulcha et al. 2018). We found that animals with mutations in *nhr-68* show reduced *srh-234p::GFP* expression when fed *E. coli* OP50 supplemented with or without exogenous vitamin B12 (Me-Cbl) compared to wild-type animals (**Figure 10E**). Feeding *nhr-68* mutants *E. coli* OP50 supplemented with both vitamin B12 (Me-Cbl) and excess propionate (40 mM) resulted in significantly higher *srh-234p::GFP* expression levels ($p<0.0001$) than compared to wild-type (**Figure 10E**). That is, adding excess propionate to *nhr-68* mutants could modestly override the repressing effects of vitamin B12 on *srh-234* expression, but not to the same extent as in wild-type animals. Thus, *nhr-68* is an important determinant in mitigating the response to excessive propionate levels on *srh-234* expression.

Together, these findings suggest that excess propionate can override the repressing effects of vitamin B12 on *srh-234* expression in ADL neurons.

MEF-2 is required for the vitamin B12-mediated reduction in *srh-234* expression

To further interrogate the mechanisms underlying the vitamin B12-dependent regulation of *srh-234* gene expression in ADL neurons, we examined candidate components and pathways. We previously reported that the MEF-2 transcription factor acts together with bHLH factors to regulate the starvation-dependent regulation of *srh-234* expression (Gruner et al., 2016). In this

mechanism, MEF-2 acts cell-autonomously with bHLH factors HLH-2/HLH-3 in ADL neurons, while HLH-30 and MLX-3 bHLH factors function in the intestine to non-cell-autonomously regulate *srh-234* expression in ADL in response to starvation signals. We found that a mutation in *mef-2* but not in *hlh-30* can fully suppress the vitamin B12-dependent reduction in *srh-234* expression when animals were fed a *Comamonas aq.* DA1877 diet, suggesting that the MEF-2 factor is required for the vitamin B12-dependent regulation of *srh-234* (**Figure 11A, 12A**). We next took advantage of *srh-234p(-MEF-2)::GFP* reporter animals in which the core of the MEF2 binding in the *srh-234* promoter is mutated (Gruner et al., 2016), and found that vitamin B12-supplemented *E. coli* OP50 bacteria fail to repress *srh-234p(-MEF-2)::GFP* expression compared to *srh-234p(WT)::GFP* expressing animals (**Figure 12B**). These results suggest that the MEF-2 factor and its binding site in the *srh-234* promoter are necessary for the vitamin B12-dependent repression of *srh-234* expression.

We next examined whether the MEF-2 binding site in the *srh-234* promoter was sufficient for the vitamin B12-dependent regulation of *srh-234*. To test this, we used a transgenic reporter strain of the *sre-1* promoter fused to *gfp* with or without the MEF-2 binding site identified in the *srh-234* promoter (AGTTATATTTAA) (**Figure 12C**) (Gruner et al., 2016). The *sre-1* promoter is specifically and highly expressed in ADL neurons, but levels of *sre-1* expression are not significantly changed in animals fed the vitamin B12-producing *Comamonas aq.* DA1877 bacteria (**Figure 4C**). Surprisingly, we found that animals carrying a transgene of the *sre-1* promoter with the inserted *srh-234*

MEF-2 site (*sre-1p(+MEF2)::GFP*) showed similar *sre-1* expression levels in ADL neurons when fed *Comamonas aq.* DA1877 compared to wild-type *sre-1p::GFP* animals (*sre-1p(WT)::GFP*) on the same diet (**Figure 11B**). These results suggest that in contrast to the starvation-dependent regulation of *srh-234* (Gruner et al., 2014), insertion of the MEF2 binding site alone is not sufficient for the vitamin B12-dependent regulation of *srh-234* expression levels in ADL neurons, suggesting the requirement of another yet unknown factor that may act together with the MEF-2 factor (**Figure 11C**).

Taken together, these findings show that the repressing effects of vitamin B12 on the expression of *srh-234* in ADL neurons is dependent on the MEF-2 transcription factor.

3. DISCUSSION

In this study, we show that the expression levels of the *srh-234* chemoreceptor gene in the ADL sensory neuron type is regulated by dietary vitamin B12. In a low vitamin B12 *E. coli* diet, *srh-234* is highly expressed in ADL but not when *C. elegans* is fed a high vitamin B12-producing *Comamonas aq.* DA1877 diet (**Figure 13**). Excess propionate and genetic perturbations in the propionate breakdown pathways are able to override the repressing effects of vitamin B12 on *srh-234* expression. In addition, the vitamin B12-mediated regulation of *srh-234* is dependent on the MEF-2 transcription factor. The mechanisms by which dietary vitamin B12 transcriptionally tunes *srh-234* could

provide *C. elegans* the means to modify long-term changes in ADL-mediated responses.

This study complements our previous work (Gruner et al., 2016; Gruner et al., 2014), which explored the dynamics in *srh-234* expression upon starvation, which was dependent on MEF-2 function and its respective MEF2 binding site present in the *cis*-regulatory sequence of *srh-234*. Similarly, we show that loss of MEF-2 function can inhibit the repression of *srh-234* expression in ADL in response to vitamin B12 produced by *Comamonas aq.* DA1877 diets, suggesting that MEF-2 has dual roles in regulating *srh-234* expression in response to both starvation and dietary vitamin B12. However, unlike starvation (Gruner et al., 2016), artificial introduction of the *srh-234* MEF2 binding site into the *cis*-regulatory sequence of the *sre-1* gene close to its E-box site that drives expression in ADL neurons (McCarroll, Li, & Bargmann, 2005) did not confer vitamin B12-induced repression via MEF-2. Instead, insertion of the *srh-234* MEF-2 site in the *sre-1* promoter showed higher *sre-1* expression levels when fed the high vitamin B12 *Comamonas aq.* DA1877 diet compared to the low vitamin B12 *E. coli* diet. One possible explanation for the increased *sre-1* expression levels in ADL is that MEF2 acts to promote (or enhance) *sre-1* transcription via its respective basic helix-loop-helix (bHLH)/E-box module after introducing the MEF2 site near the *sre-1* E-box site. In mammals, it is known that MEF2 factors can act either to activate or repress transcription depending on its interacting co-factors (Black, Ligon, Zhang, & Olson, 1996; Molkentin, Black, Martin, & Olson, 1995). Thus, MEF2 may act to repress or promote expression

based on the different bHLH factors that drive *srh-234* and *sre-1* transcription in ADL via their respective E-box sites contained in their promoters (Gruner et al., 2016; McCarroll et al., 2005).

Based on these findings, we propose a model (**Figure 13**) in which animals fed a high vitamin B12 *Comamonas aq.* DA1877 diet regulates *srh-234* expression via a transcriptional module consisting of a MEF-2 factor and its respective MEF2 binding site, together with a yet unknown factor (X) stimulated by dietary vitamin B12. This in turn may repress bHLH factors through an E-box site that promotes *srh-234* in ADL neurons via a complex mechanism involving a combination of different bHLH heterodimer pairs (Gruner et al., 2016). When animals are fed a low vitamin B12 diet of *E. coli* OP50, MEF-2 activity no longer represses *srh-234* expression in ADL. Thus, MEF-2 activity is necessary to repress *srh-234* in response to vitamin B12, but the exact nature of factor X that may act with MEF-2 remains to be determined.

In *C. elegans*, vitamin B12 is exclusively obtained from its diet, and our findings support that vitamin B12 produced by *Comamonas aq.* can repress *srh-234* expression in ADL neurons. We further showed that *E. coli* bacteria via the TonB transporter may function as a vehicle for vitamin B12 uptake by *C. elegans*, and its uptake in turn can regulate *srh-234*. However, *tonB* mutations did not completely block the repression of *srh-234* expression when vitamin B12 was added to the growth media. These findings suggest that *E. coli* may have alternative *tonB*-independent routes to obtain vitamin B12 from the media consistent with previous reports using the *acdh-1p::GFP* reporter (Revtovich et

al., 2019). Alternatively, the increased availability of exogenous vitamin B12 added to the media may be directly perceived by ADL sensory neurons, which could play additional roles in the repression of *srh-234* expression in ADL. Using olfactory assays, we showed that vitamin B12 does not appear to act as a volatile chemical cue to regulate *srh-234* in ADL neurons, but we cannot exclude the possibility that vitamin B12 is also directly sensed by ADL in the vitamin B12 supplementation experiments. Our previous work showed that elimination of all sensory inputs into ADL neurons strongly decrease *srh-234* expression in *E. coli* OP50-fed conditions similar to the effects of vitamin B12 (Gruner et al., 2014; Gruner & van der Linden, 2015), and thus it would be challenging to investigate whether physical or genetic manipulations of ADL sensory dendrites and/or cilia important for sensing the environment can alter the effects of vitamin B12 on *srh-234* expression. Future studies investigating intracellular calcium dynamics in ADL in the presence of vitamin B12 using a genetically-encoded Ca2+ sensor have the potential to reveal whether ADL neurons can also directly sense vitamin B12.

The balance between vitamin B12 and propionate levels in *C. elegans* is important for tuning *srh-234* promoter activity in ADL neurons. Mutations in the canonical (*pcca-1*, *pccb-1, mmcm-1*) and the shunt propionate (*acdh-1*, *hphd-1*) breakdown pathways, the propionate persistence detector (*nhr-68*), as well as propionate supplementation, were all able to override the repressing effects of vitamin B12 on *srh-234* expression. In mammalian models of propionic acidemia, animals lacking the propionyl CoA-carboxylase (PCCA) were found to have

elevated propionate levels shortly after birth (Miyazaki et al., 2001). Similarly, *pcca-1* mutant animals in *C. elegans* may have naturally elevated propionate levels that cannot be restored to normal levels by vitamin B12 sufficient diets alone (Watson et al., 2016). Consistent with a persistent accumulation of propionate in *C. elegans* in regulating *srh-234* promoter activity, we show that mutants of *mmcm-1*, *pcca-1*, *pccb-1* significantly enhance the levels of *srh-234* expression on an *E. coli* OP50 diet that is unable to efficiently breakdown propionate by the canonical pathway. Conversely, *srh-234* expression levels are strongly reduced in ADL when exposed to low propionate levels; for instance, in animals that are food deprived (starved) or exposed to high vitamin B12 conditions. Studies in rats demonstrated that after two days of starvation, propionate levels are rapidly decreased but again restored after re-feeding (Illman, Topping, & Trimble, 1986).

What are the functional consequences of the regulation of chemoreceptor gene expression by dietary-supplied vitamin B12? The nociceptive ADL neuron where *srh-234* is specifically expressed mediates avoidance responses to a wide variety of environmental signals such as odors (Chao, Komatsu, Fukuto, Dionne, & Hart, 2004; Troemel, Chou, Dwyer, Colbert, & Bargmann, 1995; Troemel, Kimmel, & Bargmann, 1997), pheromones (Jang et al., 2012), and heavy metals (Sambongi et al., 1999; Wen et al., 2020). Since chemoreceptor genes expressed in a specific sensory neuron type are generally linked to a common chemical response determined by the identity of the neuron in *C. elegans*, with a few exceptions in which neurons switch their preference towards odors

(Tsunozaki, Chalasani, & Bargmann, 2008), it is probable that the *srh-234* chemoreceptor may detect aversive stimuli perceived by ADL. Interestingly, vitamin B12 in mammals has anti-nociceptive properties (Erfanparast et al., 2014), and the activity of certain olfactory receptors in tissues other than neurons can respond to propionate (Pluznick et al., 2013), which is a metabolic byproduct produced by gut bacteria in mammals (Morrison & Preston, 2016). It is therefore tempting to speculate that dietary-supplied vitamin B12 can alter ADL-mediated nociceptive responses to specific cues by changing the expression levels of chemoreceptor genes. However, nothing is known about whether vitamin B12 or propionate levels affects ADL-mediated responses in *C. elegans*. Other than growth, development, and lifespan (Bito, Matsunaga, Yabuta, Kawano, & Watanabe, 2013; MacNeil et al., 2013), only recently vitamin B12 in the diet has been reported to be an important micronutrient in the regulation of predatory behaviors between nematodes (Akduman et al., 2020).

In summary, the *srh-234* chemoreceptor gene is one of a large repertoire of over 1,300 chemoreceptor genes (Robertson, 2000), many of which are localized in a relatively small subset of chemosensory neurons (Vidal et al., 2018), such that each neuron expresses multiple chemoreceptor genes. We show here dynamic changes in the expression levels of the *srh-234* chemoreceptor gene localized in the ADL sensory neuron type in response to changing dietary vitamin B12. Other studies have illustrated that dynamic expression changes in individual chemoreceptor genes can have profound effects on behavioral outcomes. For instance, changes in the expression levels

of the *odr-10* olfactory receptor required to sense diacetyl (Sengupta et al., 1996) in the male *C. elegans* contributes to its plasticity in food detection and feeding/exploration decisions in order to locate mates (Ryan et al., 2014). Further research will determine what the functional consequences are of the plasticity in *srh-234* chemoreceptor gene expression in ADL neurons in response to dietary vitamin B12.

4. MATERIAL AND METHODS

C. elegans strains and growth conditions

Strains used in this study were: wild-type N2 *C*. *elegans* variety Bristol, RB1774 *pcca-1(ok2282)*, VC1307 *pccb-1(ok1686)*, RB1434 *mmcm-1(ok1637)*, VC1011 *acdh-1(ok1489)*, RB2572 *hphd-1(ok3580)*, RB755 *metr-1(ok521)*, VC1527 *nhr-68(gk708)*, JIN1375 *hlh-30(tm1978)*, and KM134 *mef-2(gv1)*. Transgenic strains used in this study were: VDL3 *oyIs56*[*srh-234p::GFP, unc-122p::RFP*], VDL497 *sanEx497*[*sre-1p::GFP, rol-6(su1006)*], VDL494 *sanEx494*[*sre-1p(+srh-234 MEF2 site)::GFP, rol-6(su1006)*], VDL499 san*Ex499*[*srh-234p(165bp WT)::GFP, rol-6(su1006)*], VDL299 san*Ex299*[*srh-234p(165bp -MEF2)::GFP, rol-6(su1006)*], and VL749 *wwIs24*[*acdh-1p::GFP*, *unc-119(+)*]. Animals were cultivated at 20°C on the surface of Nematode Growth Media (NGM) agar. Unless specified otherwise, animals were fed *E. coli* OP50 as the primary food source (Brenner, 1974). Genotypes used in this study were

confirmed by PCR (for example, identifying deletions), or by sequencing a PCR product (for example, identifying single nucleotide changes).

Bacterial strains and growth conditions

Bacterial strains used in this study were: *E. coli* OP50, *E. coli* HT115 (DE3), *E. coli* HB101, *E. coli* BW25113, *E. coli* Δ*tonB* JW5195, *Comamonas aq.* DA1877, *Comamonas aq.* Δ*cbiA* and Δ*cbiB* mutants. Bacterial cultures were grown under standard conditions in Luria Broth (LB) media until log phase when the Optical Density (OD) 600 reached approximately 0.6. *Comamonas aq.* mutants were cultured in the presence of 100 µg/ml streptomycin plus 20 µg/ml gentamycin as a selection marker. Presence of these antibiotics did not alter the levels of *srh-234p::GFP* expression. For the killed bacteria experiments, *E. coli* OP50 bacteria were grown to log phase in LB, heat killed at 75°C for 90 minutes, and added to peptone-free NGM plates.

Measurement and quantification of promoter::*GFP* reporter expression levels

Animals carrying chemoreceptor::*GFP* reporter genes (i.e., *srh-234, sre-1*) were cultivated at 20°C on NGM plates seeded with *E*. *coli* OP50 as the bacterial food source unless indicated otherwise. Gravid adults were transferred to assay plates and removed after laying eggs. The eggs were then allowed to develop to adults. The increased rate of development when fed *Comamonas aq.* DA1877

was accounted for, and levels of promoter::*GFP* expression of adult animals were then imaged and measured under a microscope equipped with epifluorescence as previously described (Gruner et al., 2016; Gruner et al., 2014). Briefly, we mounted animals on 2% agarose pads containing 10 mM levamisole and visualized them on a Leica DM5500 compound microscope equipped with epifluorescence and a Hamamatsu CCD-camera. Microscope and camera settings were kept constant between images of different genotypes and conditions used, unless indicated otherwise. The mean pixel intensity of GFP fluorescence in the entire cell-body of ADL was quantified using Volocity software (version 6.3). Prior to measurement, images of ADL cell-bodies were cropped for promoter::*GFP* expression level analysis.

Analysis of *srh-234p::GFP* expression

To analyze *srh-234* expression in mixed bacterial diets, animals carrying the *srh-234p::GFP* reporter were exposed to mixed set ratios, i.e., 1:1, 9:1, and 99:1 ratio of *E. coli* OP50 to *Comamonas aq.* DA1877. To prepare plates, liquid bacterial cultures of OP50 and DA1877 were grown overnight at 37°C in LB broth and diluted with LB broth or concentrated to the same OD600. Bacteria were seeded onto peptone-free NGM agar plates to minimize bacterial growth. Adults expressing the *srh-234p::GFP* reporter were transferred to plates and removed after eggs were laid. Eggs were allowed to develop to adulthood in the presence

of the mixed bacterial diets, and *srh-234p::GFP* expression levels were measured and quantified as described above.

To analyze *srh-234* expression in the presence of exogenous vitamin B12 and propionic acid (aka propionate), animals carrying the *srh-234p::GFP* reporter were transferred to NGM plates seeded with *E. coli* OP50 supplemented with or without vitamin B12 (methylcobalamin or Me-Cbl, Sigma, Cat. M9756; adenosylcobalamin or Ado-Cbl, Sigma, Cat. C0884) and propionic acid (Sigma, Cat. P1386). Stocks were made in either ethanol (for Me-Cbl) and water (for Ado-Cbl and Propionic acid) to the maximum soluble concentration. Vitamin B12 and propionic acid was diluted to a final 64 nM and 40 mM concentration, respectively, in NGM agar prior to plate pouring. For *E. coli* OP50 supplementation assays with increasing Me-Cbl concentrations, we created a dilution series from a 1 mM Me-Cbl stock. To confirm vitamin B12 action, *acdh-1p::GFP* reporter animals were used as a control in parallel to the *srh-234p::GFP* expression analysis.

For bacterial olfactory assays, we exposed *srh-234p::GFP* reporter animals to a 1 cm^2 NGM agar square soaked with 1 mM Me-Cbl placed on petri dish lids. For the quadrant petri dish assay, NGM plates were seeded in each quadrant with a 100 µl spot of either *E. coli* OP50 or *Comamonas aq.* DA1877 diets. *srh-234p::GFP* reporter animals were then transferred to a single quadrant of the plate allowing only a single diet for food ingestion, while allowing olfactory cues of the surrounding diets.

Dye-filling of ADL sensory neurons

A stock dye solution prepared in DMSO containing 5 mg/ml red fluorescent lipophilic dye DiI (Sigma, Cat. 42364) was diluted in M9 buffer to a final concentration of 5 µg/ml for optimal signal intensity. Animals carrying the *srh-234p::GFP* reporter (VDL3) were soaked in DiI for one hour and then rinsed with M9 buffer twice. Stained animals were recovered for one hour on NGM plates seeded with either *E*. *coli* OP50 or *Comamonas aq.* DA1877 before examination of dye-filled ADL neurons with a Leica DM5500 microscope equipped with epifluorescence.

Analysis of ADL neuron morphology

To examine the integrity of ADL neurons, animals carrying the integrated *sre-1p*::*GFP* reporter (VDL497) were cultivated at 20°C on NGM plates seeded with *Comamonas aq.* DA1877. Briefly, we mounted animals on 2% agarose pads containing 10 mM levamisole and visualized them on a Leica Thunder 3D Tissue microscope equipped with a DFC 9000 GT/C sCMOS camera. Z-stack images were obtained at 500 ms exposure time. Small volume computational clearing (SVCC) was applied to the image data set, and a maximum intensity projection was generated.

Statistical analysis

All results are expressed as means with 95% confidence intervals. Data sets were first analyzed for Gaussian distribution using a normality test (alpha =0.05, p>0.05) using either the Shapiro-Wilk test or D'Agostino and Pearson normality test to determine whether a parametric or non-parametric statistical test should be performed. Statistical comparisons made for two groups include an unpaired *t*-test (parametric) or the Mann-Whitney *t*-test (non-parametric). For more than two groups, the ordinary one-way ANOVA (parametric) or the Kruskal-Wallis test (non-parametric) was used followed by a posthoc multiple comparisons test. Specific statistical tests and *p*-values are reported in the figure legends. All data were graphed and analyzed using Prism 9 software.

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CHAPTER III: The salt-inducible kinase KIN-29 regulates lifespan via the class II histone-deacetylase HDA-4

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1. INTRODUCTION

kin-29 encodes the *C. elegans* homolog of mammalian Salt-Inducible Kinases (SIKs). *kin-29* mutants are small, have increased propensity to develop into non-reproductive dauer larvae, have reduced chemoreceptor gene expression (Lanjuin & Sengupta, 2002; van der Linden et al., 2007), have reduced cellular ATP despite increased fat stores, and show reduced sleep (Grubbs, Lopes, et al., 2020). KIN-29 phosphorylates and inhibits the class II histone deacetylase 4 homolog HDA-4 to regulate gene expression in sensory neurons (van der Linden et al., 2007). The longevity phenotype of *kin-29* mutants is suppressed by mutations in *daf-16* (Lanjuin & Sengupta, 2002), which encodes a forkhead box protein O (FOXO) transcription factor (Kenyon, Chang, Gensch,

Rudner, & Tabtiang, 1993). These results indicated that the increased lifespan of *kin-29* mutants may be due to reduced insulin signaling.

2. RESULTS

To determine whether HDA-4 is required for the KIN-29 regulation of lifespan, we performed a survival analysis. As previously reported (Lanjuin & Sengupta, 2002), *kin-29(oy38)* mutants are long-lived. In contrast, *hda-4(oy57)* mutants are short lived (**Figure 14**). The *kin-29hda-4* double mutant longevity phenotype was similar to the phenotype of *hda-4* single mutants (**Figure 14**), suggesting that *hda-4* acts downstream of *kin-29* to regulate lifespan. Together with our previous findings (van der Linden et al., 2007), these genetic results suggest that KIN-29 may regulate lifespan via the action of HDA-4. Since SIKs can inhibit FOXO activity via HDAC4 to regulate gene expression (Mihaylova et al., 2011; B. Wang et al., 2011), it would be interesting in future studies to test the model that KIN-29/HDA-4 signaling converges on DAF-16/FOXO to modulate gene expression associated with longevity.

3. MATERIALS AND METHODS

Methods

All strains were maintained at 20°C for at least two generations before the lifespan assay. Lifespan assays were conducted at 20°C by placing age-

synchronized L4 larvae of wild-type and the indicated genotypes on 6-cm diameter plates containing nematode growth media (NGM) plates and 5 μM 5'-fluoro-2'-deoxyuridine (FUDR) (Sigma) seeded with ~10^{11} *E. coli* OP50 bacterial cells per ml and 10-15 worms per plate. The number of dead hermaphrodites was scored every two days until all worms were dead, and the percentage of survival was calculated. Death was scored as a lack of movement after a nose touch. Animals that experienced ventral rupture, bagging or walling were censored from the analysis. ~100-300 animals from each strain were used for each biological replicate. Statistical analyses and survival curves were performed with the Online Application for Survival Analysis software (OASIS 2, https://sbi.postech.ac.kr/oasis2/ (S. K. Han et al., 2016). The significance of differences in mean lifespan was calculated using the Log-Rank test.

Reagents

Strains used in this study are the wild-type strain N2 variety Bristol (Brenner, 1974), PY1476 *kin-29(oy38)*, PY4111 *hda-4(oy57)*, and PY4112 *kin-29(oy38)hda-4(oy57)* (Lanjuin & Sengupta, 2002; van der Linden et al., 2007). All strains are available at the CGC. The deletion/rearrangement in the *oy38* allele was confirmed by the polymerase chain reaction (PCR) and the single nucleotide change in *oy57* allele by sequencing of a PCR product.

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CHAPTER IV: Regulation of sleep by food and CRTC1

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ABSTRACT

Sleep and metabolism are highly interconnected, but little is known about the mechanisms underlying this link. We previously demonstrated that the *C. elegans* salt-inducible kinase (SIK) homolog KIN-29 is a key metabolic regulator of sleep. To understand how KIN-29/SIK functions, we investigated the CREB-regulated transcription co-activator CRTC1, a known SIK target. We found that *crtc-1* mutant animals are defective in movement quiescence but not feeding quiescence during stress-induced sleep (SIS). *crtc-1*, and its interacting partner *crh-1*/CREB, act upstream of EGF receptor signaling to regulate SIS. Similar to *kin-29*, loss-of-function mutations in the histone deacetylase gene *hda-4* corrects the SIS phenotype of *crtc-1* mutants, suggesting that *crtc-1* acts genetically upstream of *hda-4*. Unlike *kin-29*, the *crtc-1* mutants show a feeding quiescence phenotype similar to *daf-7* mutants and regulate *daf-7* expression, suggesting that *crtc-1* may act in food-dependent plasticity of SIS independent from *kin-29*. We propose that CRTC-1 is a new SIS regulator, and our working hypothesis is

that CRTC-1 may act both in glutamatergic neurons with KIN-29, and the DAF-7/TGF-β signaling pathways to regulate sleep.

1. INTRODUCTION

Sleep and metabolism are highly interconnected and crucial in maintaining energy and metabolic homeostasis. Sleep deprivation is associated with an increased risk of metabolic disorders including obesity, hypertension, type 2 diabetes and can lead to other serious health problems including coronary heart disease and stroke (Bedrosian, Fonken, & Nelson, 2016; Cappuccio & Miller, 2017; Gangwisch, Malaspina, Boden-Albala, & Heymsfield, 2005; Goldberg et al., 1988; Kohatsu et al., 2006; Marshall, Glozier, & Grunstein, 2008; Reich, Geyer, & Karnovsky, 1972). Sleep is a highly conserved reversible state of behavioral quiescence whose function remains a topic of debate (Crocker & Sehgal, 2010; Zimmerman, Naidoo, Raizen, & Pack, 2008). However, the link between sleep and metabolism and how these important functions are integrally regulated is still poorly understood.

Salt-inducible kinases (SIKs) are known to be highly conserved regulators of metabolism and have recently been identified to regulate sleep (Funato et al., 2016; Grubbs, Lopes, et al., 2020; Sakamoto et al., 2018). In *C. elegans*, KIN-29, a homolog of the salt-inducible kinase SIK3, was found to be a key metabolic regulator of sleep (Grubbs, Lopes, et al., 2020). Many downstream substrates have been identified to mediate the regulatory effects of SIKs including the

CREB-regulated transcription cofactors (CRTCs) and class IIa histone deacetylases (HDACs). CRTCs are directly phosphorylated by SIKs to keep them sequestered in the cytoplasm and prevent the subsequent regulation of CREB (Altarejos & Montminy, 2011; Haberland et al., 2009; Koo et al., 2005). CRTCs have been shown to be involved in a range of biological processes including energy homeostasis (Altarejos & Montminy, 2011; Y. Li et al., 2017), circadian rhythms (Sakamoto et al., 2013), cell processes (Gao et al., 2018), longevity (Mair et al., 2011), and cancers (Komiya et al., 2010). Like SIKs, there are three CRTC isoforms with CRTC-1 being the sole *C. elegans* CRTC family member (Haberland et al., 2009; Mair et al., 2011).

Similar to CRTCs, HDAC4, a member of the class IIa histone deacetylases, are localized to the nucleus as a result of SIK-mediated phosphorylation (Mihaylova et al., 2011). HDAC4 has a highly conserved binding site for the MEF2 transcription factor (Darling & Cohen, 2021; Haberland et al., 2009; Miska et al., 1999; van der Linden et al., 2007). Regulation by HDAC4 can convert MEF2 from a transcriptional repressor to an activator and provides a mechanism for external signal and transcriptional integration (Lu et al., 2000). HDAC4 is abundantly found in the brain and skeletal growth plates and plays a key role in the formation of the skeleton and functions in the nervous system (Berdeaux et al., 2007; Chen et al., 2015; McKinsey et al., 2001; Miska et al., 1999; van der Linden et al., 2007). Despite both SIK-HDAC4 and SIK-CRTC signaling playing a key role in the regulation of gene expression in multiple cell

types, much is still to be determined about how these pathways interact in sleep-metabolic interactions mediated by SIK signaling.

The nematode *C. elegans* is a powerful model to understand the mechanisms underlying SIK signaling in sleep and metabolism. *C. elegans* satisfies all the behavioral requirements of sleep and shares extensive homology with mammalian genes that regulate sleep and metabolism (Iwanir et al., 2013; Nagy et al., 2014; Trojanowski & Raizen, 2016; Watts & Ristow, 2017). *C. elegans* exhibits a period of quiescence following exposure to noxious conditions like heat or UV-irradiation called stress-induced sleep (SIS) (Hill et al., 2014; Iannacone et al., 2017; Nelson et al., 2014). This sleep state is represented by animals displaying a lack of feeding, reduced motor activity, and an increased arousal threshold as seen in mammalian sleep (Hill et al., 2014). SIS appears to be beneficial and presents as a protective state to repair cellular damage (Hill et al., 2014; Jones & Candido, 1999). *C. elegans* has an extensive toolkit to assess fat stores, metabolic activity, and measure sleep activity making it an excellent model to understand how sleep and metabolism are integrated.

SIS is dependent on epidermal growth factor (EGF) which is known to promote sleep in mammals (Kramer et al., 2001; Kushikata et al., 1998; Van Buskirk & Sternberg, 2007). EGF and its receptor (EGFR), are expressed rhythmically in the brain to reduce behavioral activity and induce drowsiness (Kramer et al., 2001; Kushikata et al., 1998). Like the mammalian hypothalamus, the *C. elegans* sensory nervous system acts as a command center to regulate sleep (Bringmann, 2018; Konietzka et al., 2020b). The *C. elegans* lone homologs

for EGF and EGFR are LIN-3 and LET-23, respectively (Aroian, Koga, Mendel, Ohshima, & Sternberg, 1990; Hill et al., 2014). In response to cellular stress, *C. elegans* display increased sleep in part as a result of EGF signaling and downstream activation of sleep promoting neuron ALA (Nath, Chow, Wang, Schwarz, & Sternberg, 2016; Van Buskirk & Sternberg, 2007).

Another important growth factor that modulates *C. elegans* sleep is DAF-7, a transforming growth factor beta (TGF-β), which is exclusively secreted from the ASI sensory neuron in response to environmental stimuli like food, pheromone, and pathogen signals (Meisel et al., 2014; Schackwitz, Inoue, & Thomas, 1996). Similar to mammalian systems (Colak & Ten Dijke, 2017; Itoh et al., 2014; Penn, Grobbelaar, & Rolfe, 2012; Sanjabi, Oh, & Li, 2017; Watabe & Miyazono, 2009; Wu & Hill, 2009; Yoshimura, Wakabayashi, & Mori, 2010), the DAF-7/TGF-β pathway regulates a wide array of biological functions in *C. elegans*. One such pathway is the food-dependent plasticity pathway of sleep where quiescence is exhibited postprandially following a fast. In this pathway, food deprivation suppresses SIS by reducing sleep need and is mediated by the *daf-7*/TGF-β pathway, functioning upstream of the ALA neuron (Goetting et al., 2018). Food deprivation has been well documented to have a profound suppressing effect on sleep from invertebrates to humans (Goetting et al., 2018; Jacobs & McGinty, 1971; Keene et al., 2010; MacFadyen, Oswald, & Lewis, 1973).

Here, we investigated the *C. elegans* homolog of the CREB-regulated transcription co-activator CRTC-1, a known target of SIKs. We show that *crtc-1*

mutant animals are defective in movement quiescence during SIS. The reduced sleep phenotype of *crtc-1* mutants can be rescued by overexpressing *crtc-1* controlled by its own promoter. Two mutant alleles of *crh-1*, the CREB homolog known to interact with CRTC1, also have reduced SIS as shown previously (Cianciulli et al., 2019). We find that loss of function mutations in the class II HDAC homolog *hda-4* correct the SIS phenotype of *crtc-1* mutants, suggesting *crtc-1* may act together with *hda-4* to regulate SIS downstream of KIN-29. SIS is regulated by EGF and modulated by the food-dependent DAF-7/TGF-β pathway. We show that both *crtc-1* and *crh-1* act upstream of EGF receptor signaling in the ALA/RIS neurons to regulate SIS. However, unlike in *kin-29* mutants, we show that *crtc-1* mutations strongly reduced *daf-7*/TGF-β expression in the ASI neurons, and show feeding quiescence similar to *daf-7* mutants. Together, these findings suggest that CRTC-1 may act both within the KIN-29/SIK signaling pathway and DAF-7/TGF-β food-dependent pathway as a new regulator of SIS.

2. RESULTS

CRTC-1 and CRH-1 are required for movement quiescence after UV exposure

CRTC-1 is a known downstream target of SIK that has been shown to be involved in metabolism (Altarejos & Montminy, 2011; Darling & Cohen, 2021). We hypothesized that CRTC-1 in *C. elegans* may also be involved in the regulation of sleep by KIN-29/SIK. To determine if *crtc-1* regulates SIS, we

measured movement quiescence in *crtc-1(tm2869)* loss-of-function and *crtc-1(wbm57)* null mutant animals. We exposed animals to UV radiation and continuously monitored them for 8hrs on WorMotel chips (see methods for additional details). We also measured total minutes of quiescence during the first which was cloned into the second multiple cloning site (MCS) of the pMC70 plasmid containing an *unc-54* 3'UTR sequence under the control of the *crtc-1* (3.0 kb) promoter. This construct was injected into wild-type worms to create transgenic animals expressing *crtc-1* cDNA under the control of its own promoter fused to GFP. These transgenic animals were crossed with *crtc-1* loss-of-function mutants and movement quiescence was measured. We found that *crtc-1* expressed under the control of its own promoter was able to restore the reduced SIS phenotype of *crtc-1* mutant animals to wild-type levels (**Figure 15A-B**).

Next, we wanted to explore the role of CRTC1's interacting partner CREB. The *C. elegans* CREB homolog *crh-1* was previously shown to have reduced movement quiescence suggesting *crh-1* is required for SIS (Cianciulli et al., 2019). We also measured movement quiescence of *crh-1* mutant animals and confirmed that both *crh-1(tz2)* and *crh-1(n3315)* mutant animals display reduced SIS phenotypes following UV stress (**Figure 15E-F**). These data suggest that CRTC-1 and CRH-1 are required for movement quiescence after UV exposure.

CRTCs interact with CREB through a conserved N-terminal binding domain (Conkright et al., 2003). The CREB-binding-domain (CBD) of CRTC-1 is critical for its ability to associate with its cofactor CREB (Luo et al., 2012). It was recently shown that a CBD point mutation can fully suppress lifespan extension

of *set-2* long-lived mutants in *C. elegans* (Silva-García et al., 2022). This suggests that CRTC-1 fulfills its role as a co-transcriptional activator of CREB through its CBD domain in the regulation of longevity (Silva-García et al., 2022). We wanted to determine if mutations in the CBD site could similarly suppress movement quiescence and regulate SIS. We exposed *crtc-1* CBD mutant animals to UV irradiation and again measured movement quiescence over 8 hours and during the first 4 hours following UV shock. We found that *crtc-1* CBD mutant animals also have reduced movement quiescence (**Figure 15C-D**). This suggests that the CREB-binding (CBD) domain of CRTC-1 is important for SIS regulation.

CRTC-1 and CRH-1 act upstream of EGF to regulate SIS

Previously, we determined that *kin-29* functions upstream of EGF signaling (Grubbs, Lopes, et al., 2020). We then asked if *crtc-1* and its interacting partner *crh-1* also act upstream of EGF. To determine whether CRTC-1 acts upstream or downstream of EGF and ALA activation in the SIS pathway, we overexpressed LIN-3/EGF in *crtc-1* and *crh-1* mutants. To induce LIN-3/EGF overexpression, animals expressing a *Phsp-16.2*::*LIN-3C* transgene were heat-shocked for 30 min. We then measured total minutes of EGF-induced quiescence on a WorMotel chip between hours 2-4 during peak EGF-quiescence (see methods for details). We observed no effect on quiescence-induced by EGF overexpression in *crtc-1* mutants (**Figure 16A**). Similarly, we tested *crh-1* mutant

animals and did not find attenuation of EGF induced quiescence in either the *crh-1(tz2)* (**Figure 16B**) nor the *crh-1(n3315)* alleles (**Figure 16C**). These results were consistent with findings from *kin-29* mutant animals (Grubbs, Lopes, et al., 2020). Together, the findings suggest that *crtc-1* and *crh-1* may be acting upstream of EGF and ALA activation to regulate sleep.

HDA-4 acts downstream of CRTC-1 to regulate SIS

The class II histone deacetylase *hda-4* was previously shown to act downstream of *kin-29* in sensory neurons to control metabolic regulation of sleep (Grubbs, Lopes, et al., 2020). To determine if HDA-4 is similarly required in the CRTC-1 regulation of sleep, we measured the SIS response of animals of *crtc-1; hda-4* loss of function double mutants. Mutations in *hda-4* restored the defective SIS sleep phenotype of *kin-29* mutant animals. Measuring movement quiescence over 8 hours (**Figure 17A**) and the first 4 hours (**Figure 17B**) after UV shock, we now find that loss of function mutations in *hda-4* also correct the reduced SIS phenotype of *crtc-1* mutant animals. This indicates that *hda-4* acts downstream of both *kin-29* and *crtc-1* to regulate sleep. We next tested the movement quiescence of double mutant animals of *crtc-1*; *kin-29* (**Figure 17C-D**) and found an even more severe reduction of SIS, suggesting that *crtc-1* may also be acting in another pathway parallel of KIN-29/EGF (see feeding quiescence results below). These results suggest that *crtc-1* and *hda-4* may act together to regulate SIS downstream of KIN-29.

crtc-1 mutations downregulate *daf-7* expression in ASI neurons

The DAF-7/TGF-β signaling pathway mediates food-dependent plasticity of SIS. Animal with mutations in *daf-7* display a reduced movement quiescence phenotype (Goetting et al., 2018). Another method of quantifying quiescence is through assessment of feeding rate. Quiescent animals display little to no feeding as measured by pharyngeal pumps. A pump is defined a backward movement of the pharyngeal grinder, the tooth-like structure in the terminal bulb of the pharynx. *daf-7* mutants show a less severe feeding quiescence phenotype compared to movement quiescence (Goetting et al., 2018). Previously, it was shown that *kin-29* mutant animals do not display a reduced feeding rate following UV stress. We wanted to determine if *crtc-1* mutant animals had a similar feeding quiescence phenotype. To test this, we exposed animals to UV radiation and measured feeding quiescence two hours later by observing the number of pharyngeal pumps over 10 seconds (see methods for details). Animals were considered feeding quiescent if no pumps were measured during a 10 second observation window. We found that unlike *kin-29* mutant animals, mutants of *crtc-1* displayed a less severe feeding quiescence phenotype (**Figure 18A**). This, combined with the movement quiescence findings of suggests that *crtc-1* may also be acting in a parallel pathway to regulate SIS.

It has been previously shown that *kin-29* does not regulate *daf-7*/TGF-β expression in the ASI neuron of *C. elegans* (Lanjuin & Sengupta, 2002), which

we verified (**Figure 18B**). Since *crtc-1* mutants display a less severe feeding quiescence phenotype compared to movement quiescence in a manner similar to *daf-7* mutants, we wanted to determine whether *crtc-1* affects *daf-7* expression. Using an integrated *daf-7p::gfp* reporter, we found that *daf-7* expression in ASI neurons is strongly reduced by mutations in *crtc-1* (**Figure 18D**). Interestingly, mutations in *crh-1*/CREB that interacts with *crtc-1* also regulate *daf-7* expression levels (J. Park et al., 2021). Moreover, it was reported that CRH-1 binds upstream of *daf-7*/TGF-β to promote *daf-7* expression in ASI neurons (J. Park et al., 2021), suggesting that CRTC-1 via CRH-1 may directly regulate *daf-7* expression.

Together, these data support the hypothesis that CRTC-1/CRH-1 act both in the KIN-29/SIK-dependent EGF signaling pathway, as well as in the DAF-7/TGF-β food-dependent pathway to regulate SIS.

3. DISCUSSION

Sleep is a highly conserved state that likely aids survival, but the molecular mechanisms and function of sleep remain a mystery (Hill et al., 2014; Krueger, Frank, Wisor, & Roy, 2016). This work builds on our previous study (Grubbs, Lopes, et al., 2020), which explored the role of *kin-29* in the metabolic regulation of sleep. KIN-29 plays a role in both sleep regulation and fat mobilization in a subset of glutamatergic neurons in *C. elegans* supporting a mechanistic link between sleep and metabolism (Grubbs, Lopes, et al., 2020).

SIS is mediated by the KIN-29 signaling upstream of EGF (Fry, Laboy, Huang, Hart, & Norman, 2016; Grubbs, Lopes, et al., 2020; Konietzka et al., 2020a; Van Buskirk & Sternberg, 2007) and the DAF-7/TGF-β pathways (Goetting et al., 2018; You et al., 2008). CRTC-1 appears to be a new regulator of SIS that is involved in both growth factor pathways, but the exact site of action is still unknown. CRTC-1 is widely expressed and may act in various tissues and multiple pathways. Our data suggest that CRTC-1 and its interactor CRH-1 act with the KIN-29/SIK-dependent pathway upstream of EGF. In addition, we found that CRTC-1/CRH-1 regulate *daf-7* expression suggesting it may also act in parallel with the KIN-29/SIK-independent food-dependent plasticity pathway mediated by DAF-7/TGF-β. This pathway is known to modulate sleep downstream of ALA/RIS sleep promoting neurons (Goetting et al., 2018). We propose the following potential model of the CRTC-1/CRH-1 regulation of SIS. In response to cellular stress, CRTC-1 and CRH-1 may act in glutamatergic

neurons with KIN-29/SIK to mobilize fat stores upstream of EGF and sleep promoting neurons ALA/RIS. CRTC-1/CRH-1 may also act independent of KIN-29/SIK in the food-dependent plasticity pathway of sleep mediated by DAF-7/TGF-β (**Figure 19**). While HDA-4 appears to act downstream of CRTC-1 the exact nature of the relationship between KIN-29, HDA-4 and CRTC-1/CREB is unclear. Recent work in mice has shown that HDAC4 regulates non-rapid eye movement sleep (NREMS) in a CREB dependent manner (R. Zhou et al., 2022) suggesting that HDAC4 coordinates with CREB downstream of SIK3 to regulate common targets downstream of SIK3 to regulate sleep. Interestingly, these results challenge previous reports of HDAC4 suppressing CREB activity (J. Li et al., 2012; Sen & Sen, 2016). This HDAC4 and CREB coordination may similarly act in *C. elegans* to regulate sleep downstream of KIN-29.

Identifying the CRTC-1/CRH-1 site of action will reveal how these players function together in sleep and metabolic gene regulation. To uncover this, we can restore CRTC-1 and CRH-1 specifically in the *odr-4* expressing glutamatergic neurons that KIN-29 acts, in an attempt to rescue the *crtc-1/crh-1* reduced SIS phenotypes. Placing *crtc-1* and *crh-1* under the control of an *odr-4* promoter we can rescue the defective movement quiescence phenotypes of *crtc-1* and *crh-1* mutant animals. A similar method can be used to investigate the CRTC-1/CRH-1 site of action in the DAF-7/TGF-β regulation of SIS. For instance, the reduced SIS phenotype of *kin-29* mutants cannot be rescued by introduction of *kin-29* function specifically in ASI (Grubbs, Lopes, et al., 2020) but it is unknown if the same is true for *crtc-1*. We plan to test this by placing either *crtc-1* and *crh-1*

under the control of an ASI specific promoter in *crtc-1* or *crh-1* mutant animals, respectively, to rescue their reduced movement quiescence phenotypes.

The findings of this work provide insight into the mechanistic link between sleep, feeding, and metabolism. Understanding the regulation of stress-induced sleep in *C. elegans* can help uncover the function and evolution of sickness sleep.

4. MATERIALS AND METHODS

C. elegans strains and growth conditions

Worms were grown at 20°C under standard conditions on nematode growth medium (NGM) with *E. coli* OP50 as the primary food source as previously described (Brenner, 1974). The wild-type strain used was *C. elegans* variety Bristol, strain N2. Mutant strains used in this study were: PY1479 *kin-29(oy38)*, VDL320 *crtc-1(tm2869)*, YT17 *crh-1(tz2)*, MT9973 *crh-1(n3315),* PY4111 *hda-4(oy57)*, WBM1041 *crtc-1(wbm18),* WBM1254 *crtc-1(wbm57)*. Transgenic strains used in this study were: PS5009 *pha-1(e2132ts)III; him-5(e1490)V; syEx723[hs::LIN-3C; myo2:GFP; pha-1(+)]*, PY6815 *oyIs75[crtc-1::crtc-1::GFP + unc-122::RFP],* PY7606 *crtc-1(tm2869); Is[daf-7::GFP, rol-6],* VDL438 *kin-29(oy38); Is[daf-7p::GFP +rol-6].* Animals were cultivated on the surface of Nematode Growth Media (NGM) agar at 20°C and fed *E. coli* OP50 as the primary food source (Brenner, 1974). Genotypes used in this study were confirmed by PCR (for example, identifying deletions), or by sequencing a PCR

product (for example, identifying single nucleotide changes). Double mutant strains were constructed using standard genetic methods, and the presence of each mutation was confirmed by PCR or sequencing.

SIS induction by UV irradiation

UV-induced sleep assays were performed as previously described (Grubbs et al 2020). First-day-old adult animals were exposed to 1,500J/m2 UVC irradiation (254 nm) in a Spectrolinker XL-1500 (Spectroline). Animals were housed on a WorMotel chip placed in an uncovered plastic 5.5cm petri dish. Wells were filled with NGM agar and spread with a thin layer of *E. coli* OP50 bacteria immediately before experiment unless otherwise indicated.

Movement quiescence assessment

Movement quiescence was measured using a 48-well (6 x 8) WorMotel chip (Churgin & Fang-Yen, 2015). First-day-old adult animals were exposed to UV shock as described above on normal NGM agar spread with a thin layer of *E. coli* OP50 unless otherwise indicated. Immediately following UV exposure, WorMotel chips were placed in petri dishes with moistened Kimwipes to maintain humidity and lids treated with a thin layer of 20% TWEEN® 20 to prevent condensation before parafilmed and imaged under dark-field illumination by red LED lighting. Images were captured continuously every 10 s for 8 hours and analyzed by frame subtraction using a custom MATLAB script (Churgin et al.,

2019). Quiescence was defined as a lack of pixel change between frames and missing worms were censored from analysis (Raizen et al., 2008).

Feeding quiescence assessment

Animals were placed on NGM plates freshly seeded with *E. coli* OP50 and exposed to UV shock as previously described. Treated animals were left undisturbed for 2 hours. Feeding quiescence was then measured by manual counting of pharyngeal pumps under direct observation through stereomicroscope under 40x-115x magnification over 10 seconds. A pharyngeal pump was counted as a single backward movement of the pharyngeal grinder, the tooth-like structure in the terminal bulb of the pharynx. Number of pumps was quantified, and non-pumping animals were considered feeding quiescent.

Induction of EGF

EGF induction assays were performed as previously described (Grubbs et al 2020). First-day-old adult animals carrying *Phsp-16.2::lin3C* transgene were housed on the NGM agar surface seeded with a thin layer of *E. coli* OP50 bacteria of a WorMotel chip placed in a 5.5cm petri dish and sealed with parafilm. The dish was fully submerged in a 33°C water bath for 30min. After heat-induced transgene induction, movement quiescence was measured continuously for 8 hours as previously described. EGF-induced quiescence was quantified between hours 2-4 after heat exposure.

Statistical analysis

Data sets were first analyzed for Gaussian distribution by normality test (alpha =0.05, p>0.05) using either the Shapiro-Wilk test or D'Agostino and Pearson normality test to determine whether a parametric or non-parametric statistical test should be performed. Statistical comparisons made for two groups include an unpaired *t*-test (parametric) or the Mann-Whitney *t*-test (non-parametric). For more than two groups, the ordinary one-way ANOVA (parametric) or the Kruskal-Wallis test (non-parametric) was used followed by a posthoc multiple comparisons test. Specific statistical tests and *p*-values are reported in the figure legends. All data were graphed and analyzed using Prism 9 software.

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CONCLUDING REMARKS

The work presented in this dissertation provides important insight into understanding the mechanisms of animal-environment interactions. The goal of this dissertation was to provide further comprehension into the mechanisms of SIK to understand how internal and environmental signals regulate gene expression and behavior. The role of SIKs have been examined in a myriad of biological functions including gene expression, longevity, metabolism, and sleep (Grubbs, Lopes, et al., 2020; Katoh et al., 2004; Patel et al., 2014; Sun et al., 2020; van der Linden et al., 2007; Z. Wang et al., 1999). I show that the KIN-29/SIK kinase acting in a subset of sensory neurons regulates chemosensory behaviors, lifespan, and sleep pathways to allow organisms to properly respond and interact with their environments.

In **Chapter 2** I demonstrated that the KIN-29/SIK-dependent chemoreceptor gene *srh-234* is not only regulated by feeding state but also by diet. Diet-derived vitamin B12 represses *srh-234* expression in the ADL neuron when supplied by both B12 producing bacterial diets like *Comamonas* and exogenous supplementation to B12 deficient *E. coli* diets. In the latter case, bacteria like *E. coli* may supply vitamin B12 to the animal by functioning as a nutrient uptake vehicle. The regulatory mechanism of vitamin B12 suggest the diet-dependent effect is distinct from the starvation response (Gruner et al., 2014). Like in the case of starvation, *srh-234* expression in ADL is dependent on the function of MEF-2 and its binding site (Gruner et al., 2014). Conversely, while

artificial insertion of the *srh-234* MEF2 binding site successfully reduced expression of another ADL expressed chemoreceptor gene, *sre-1*, in starvation, *sre-1* expression in the diet effect instead showed an increase in expression levels (Gruner et al., 2016). This surprising result could be due to a difference of bHLH factors that drive expression of each chemoreceptor gene (Gruner et al., 2016; McCarroll et al., 2005). Our model (**Figure 13**) proposes that dietary vitamin B12 regulates *srh-234* expression through MEF-2 and its respective binding site together with an unknown factor (X). The exact nature of this factor (X) that may act with MEF-2 is still to be characterized but our findings suggest MEF-2 is necessary to repress *srh-234* expression in response to vitamin B12. Future studies to identify and characterize factor (X) can be done using a forward genetic screen, a major advantage of the *C. elegans* model system. Using the *srh-234p::gfp* reporter strain, we can isolate animals that do not show reduced *srh-234p::gfp* expression in the presence of vitamin B12. These individuals can then be genetically sequenced to identify new genes required for the dietary vitamin B12 suppression of *srh-234* expression. We could then also find instances in which MEF-2 is enriched or suppressed to determine if there is more to the role of MEF-2 in this regulation of gene expression. Further, while the importance of dietary vitamin B12 is highly apparent, the exact functional role of this micronutrient is yet to be determined. Preliminary findings suggest that vitamin B12 may influence additional biological functions like sleep (data not shown). Moreover, the function of the *srh-234* chemoreceptor gene itself is not known. A member of the serpentine receptor family, *srh-234* is logically predicted

to be involved in G protein-coupled receptor signaling to help transduce external signals into internal responses (van der Linden et al., 2007). As mentioned in the chapter above, *srh-234* may detect aversive stimuli based on the nociceptive identity of the ADL neuron of *C. elegans.* This forward genetic screen may shed light on both the functions and identities of factor (X) and the *srh-234* chemoreceptor gene.

In **Chapter 3**, I show that KIN-29/SIK is involved in the regulation of lifespan via the downstream target HDA-4. *kin-29* mutants are long lived while *hda-4* mutants are short lived. Double mutants of *kin-29* and *hda-4* also show a reduced lifespan suggesting *hda-4* acts downstream of *kin-29* to regulate lifespan. Here, I demonstrate a role for KIN-29/SIK in aging and longevity that has not been previously determined. Another downstream target of SIK, CRTC-1, described in the regulation of sleep in Chapter 4, is well documented to be a regulator of aging and longevity (Mair et al., 2011). However, it is not known if or how CRTC-1 regulates lifespan in the KIN-29/SIK pathway. It would be logical to explore the role of CRTC-1 and KIN-29/SIK in the regulation of aging in future studies. These results again demonstrate KIN-29/SIK acts to regulate multiple biological pathways.

My work presented in **Chapter 4** identified CRTC-1 to be a new regulator of sleep that may work with both the DAF-7/TGF-β food-dependent plasticity pathway and the KIN-29/SIK and EGF signaling pathways in the regulation of sleep. CRTC-1 and its interacting partner CRH-1/CREB appear to act with both of these pathways as evidenced by genetic mutants. *crtc-1* mutants display a

reduced movement quiescence phenotype similar to that of *kin-29* mutants but a feeding quiescence phenotype more reminiscent of *daf-7* mutant animals (**Figure 18A**). While *hda-4* appears to act downstream of *crtc-1,* it is unclear if they are similarly regulated by upstream affecters like SIK and AMPK. The exact site of action of *crtc-1* and its interactor *crh-1* is still uncertain.

Activation of AMPK plays and important role in maintaining and restoring intracellular ATP levels (Garcia & Shaw, 2017). In mammals, phosphorylation of AMPK is lower during sleep and SIK3 gain-of-function mice have elevated phospho-AMPK levels (Scharf, Naidoo, Zimmerman, & Pack, 2008; Z. Wang et al., 2018). Consistent with these results, *kin-29* loss of function mutants display low levels of phospho-AMPK (Grubbs, Lopes, et al., 2020). CRTCs are also direct targets of AMPK and have been shown to be involved in regulating lifespan (Mair et al., 2011). Similar to KIN-29/SIK, AMPK phosphorylation causes cytosolic sequestering and subsequent inactivation of CRTC-1 and downstream partner CREB (Mair et al., 2011). This mechanism could potentially also be involved in the regulation of SIS. However, stressors like starvation and heat-stress, conditions known to activate AMPK, can induce translocation of CRTC-1 to the cytosol (Mair et al., 2011). Therefore, it is important to identify the localization of CRTC-1 during SIS to further understand its role. Moreover, it has been found that the CRTC/CREB pathway is critically involved in mitochondrial biogenesis (Altarejos & Montminy, 2011). Depletion of cellular ATP is sufficient to upregulate CRTC-CREB activity and promote mitochondrial gene expression (Altarejos & Montminy, 2011). However, sleep is associated with overall reduced

levels of ATP, thus similarly testing ATP levels and fat stores of *crtc-1/crh-1* mutants as previously done in *kin-29* mutants may shed light on this metabolic regulation of sleep. Lastly, CRTC-1 has two AMPK phosphorylation sites that when mutated causes CRTC-1 to be constitutively active in the nucleus (Mair et al., 2011). In aging and longevity, mutations of both sites lead to reduction of lifespan, but it is not known how these phosphorylation sites affect sleep. Based on these findings in aging, we can hypothesize that it may be CRTC-1 nucleo-cytoplasmic shuttling regulates sleep. Therefore, as previously mentioned, if *crtc-1* is required in ASI to regulate SIS, we can test this hypothesis that the nucleo-cytoplasmic shuttling mechanism of CRTC-1 regulates sleep in a single neuron.

Recent work suggests that KIN-29 functions in sensory neurons during the time of sleep acting as an energy sensor to promote sleep rather than in a developmental manner (Grubbs, van der Linden, & Raizen, 2020). Using an auxin inducible degron transgenic strain crossed with *kin-29* mutant animals, exposure to the phytohormone auxin induces rapid and reversible degradation of KIN-29 (Grubbs, van der Linden, et al., 2020; Ruegger et al., 1998; L. Zhang, Ward, Cheng, & Dernburg, 2015). This system could potentially be used with CRTC-1 to temporally and spatially observe CRTC-1 function during SIS.

Again, sleep and metabolism are highly interconnected, and these sleep regulatory pathways found in *C. elegans*. For instance, in obese melanoma cancer patients, increased TGF-β levels correlated with melanoma aggressiveness and severity of obstructive sleep apnea but was attenuated when leptin levels were high with suggesting a homeostatic response between sleep

and metabolism (Cubillos-Zapata et al., 2020). This complicated finding encompasses the TGF-β pathway, cancer, sleep, and metabolic function each of which can be further studied in *C. elegans* to establish a causal mechanism of these associations.

While not discussed in depth in this dissertation, understanding the role of diet in the food and CRTC-1 regulation of sleep presents as an exciting future avenue of exploration. Diet affects fat stores in *C. elegans* (Brooks, Liang, & Watts, 2009), therefore it is logical to think it is possible that diet modulates sleep in other ways. The variability in diet composition as well as the diet-induced changes in life history traits suggests there may also be a link between diet and sleep. Sleep-deprived fruit flies show a dramatic build-up of ROS in the intestine that accumulates and results in premature death suggesting a restorative role of sleep in relation to gut function (Vaccaro et al., 2020). Diet exerts great influence over *C. elegans* physiology affecting life history traits such as metabolism (Brooks et al., 2009; Coolon et al., 2009; Soukas et al., 2009; Watson et al., 2013), development (Soukas et al., 2009), and aging (MacNeil et al., 2013; Shtonda & Avery, 2006). As bacterivores *C. elegans* obtains all of its nutrients from its diet and each bacterial diet has a unique nutritional composition (Coolon et al., 2009; Watson et al., 2013; Watson et al., 2014a). For example, two common laboratory *E. coli* diets OP50 and HT115 have relatively equal protein and fat levels, but HT115 diets contain a higher level of carbohydrates compared to OP50 and worms fed the HT115 diet show differential fat stores (Brooks et al., 2009). Diets also vary in micronutrient levels as exemplified by the *Comamonas*

aquatica DA1877 diet which produces vitamin B12 that provides nutrition and acts as a cofactor in metabolic reactions (Watson et al., 2014a). The diversity of these bacterial diets is analogous to variability in mammalian diets in both nutritional composition and the effect on physiology. The balance of satisfying the need to sleep and the foraging activity requires an integration of internal state signals and external environment signals to generate an optimal behavioral response. The SIK signaling pathway may be a key mechanism in coordinating this behavior.

In summary, the work presented in this dissertation demonstrates that the convergence of environmental and internal diet, stress, and hunger signals in a subset of glutamatergic sensory neurons where the SIK kinase KIN-29 acts, may be one mechanism by which animals are able to coordinate gene expression, development, and behavior. While KIN-29 is expressed in this subset of sensory neurons, it may be differentially functioning in each of these neurons. KIN-29 may act as a conditional modulator depending on which signals are received by which neurons to ultimately regulate chemosensory behaviors, development and sleep. Despite its high level of conservation across organisms the function of sleep remains heavily debated and sleep disruption and deprivation are proven to be highly detrimental, even lethal (Jung et al., 2011; Zhao et al., 2017). Research using SIS as a model will further our understanding of the function and regulation of normal healthy sleep. Studies on internal state such as endocrine and metabolic signaling mechanisms can further our understanding of the functions of sensory neural circuits (Kanwal et al., 2021). While *C. elegans* is in

excellent model for laying the groundwork for SIK signal integration, the high degree of conservation across organisms suggests that SIK regulation in other model systems can be investigated further.

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TABLES

Table 1. Similarities of sleep between *C. elegans* and other species

Shared Trait	Mammals	*Drosophila*	*C. elegans*
Reversible quiescent behavior	YES	YES	YES (Raizen et al., 2008)
Increased arousal threshold	YES	YES	YES (Raizen et al., 2008)
Stereotypical posture	YES	YES	YES (Iwanir et al., 2013)
Homeostatic response to sleep deprivation	YES	YES	YES (Nagy et al., 2014)
Sleep deprivation may be lethal	YES	YES	YES (Raizen et al., 2008)
EGF signaling promotes sleep	YES	YES	YES (Raizen et al., 2008)
SIK promotes sleep	YES	YES	YES (Raizen et al., 2008)
HDAC4 inhibits sleep	YES	?	YES (Grubbs, Lopes, et al., 2020)

FIGURES

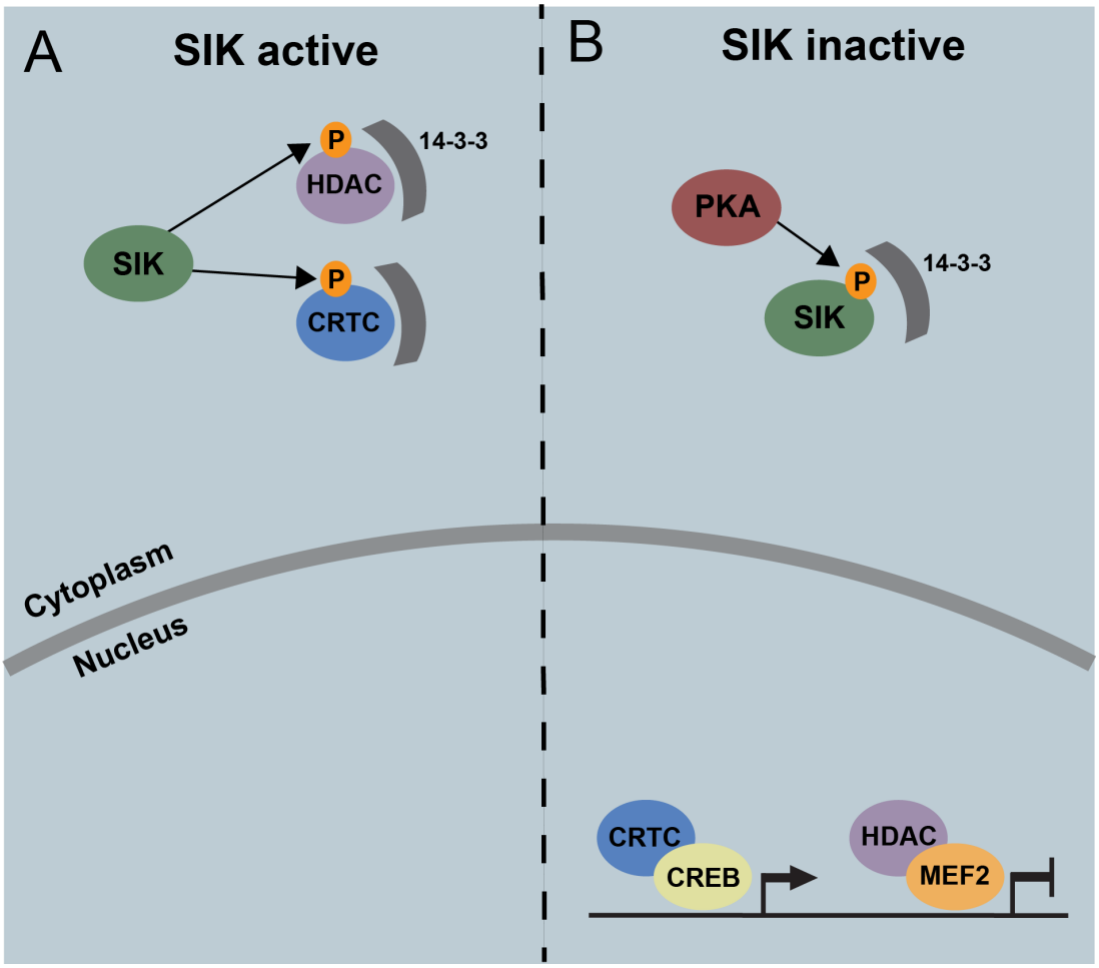


Figure 1. Model of SIK phosphorylation activity.

(A) SIK phosphorylates downstream targets HDAC and CRTC causing them to bind to 14-3-3 regulatory proteins and remain in the cytoplasm. **(B)** SIK is phosphorylated by PKA causing it to be sequestered by 14-3-3 regulatory proteins and become inactive. When inactivated, SIK is unable to phosphorylate downstream substrates HDAC and CRTC allowing translocation to the nucleus where CRTC promotes CREB transcription and HDAC inhibits MEF2 transcription. Adapted from (Darling & Cohen, 2021).

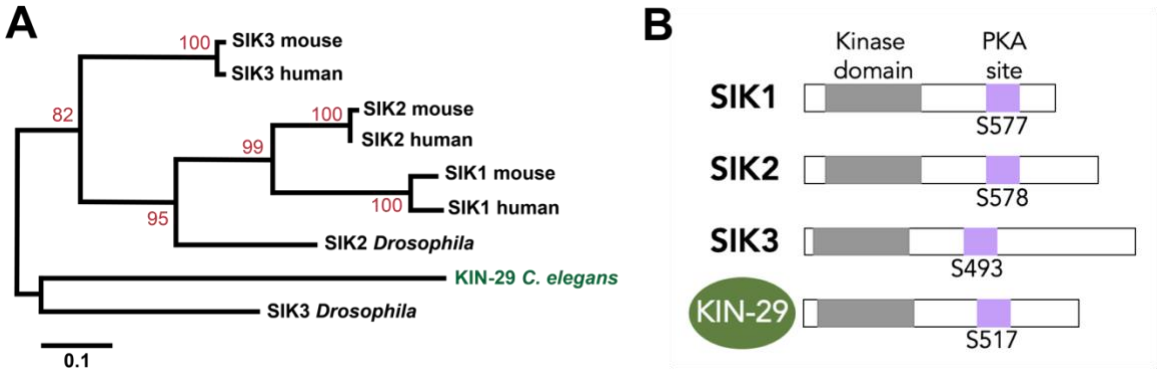


Figure 2. Conservation of SIK.

(A) SIK phylogenetic tree adapted from (Grubbs, Lopes, et al., 2020). Values denoted in red are the bootstrap values in percentages of each branch. Alignments were performed with a maximum likelihood method MEGA (Kumar, Stecher, Li, Knyaz, & Tamura, 2018) (www.megasoftware.net). Sequences (UniProtKB) used were as follows: SIK3 *Drosophila* (Q4QQA7), SIK2 *Drosophila* (O77268), SIK1 mouse (Q60670), SIK2 mouse (Q8CFH6), SIK3 mouse (Q6P4S6), SIK1 human (P57059), SIK2 human (Q9H0K1), SIK3 human (Q9Y2K2) and KIN-29 *C. elegans* (Q21017). Poorly aligned sequence regions (N- and C-terminal parts) were removed. **(B)** Conserved SIK kinase domain and PKA sites adapted from (Lanjuin & Sengupta, 2002).

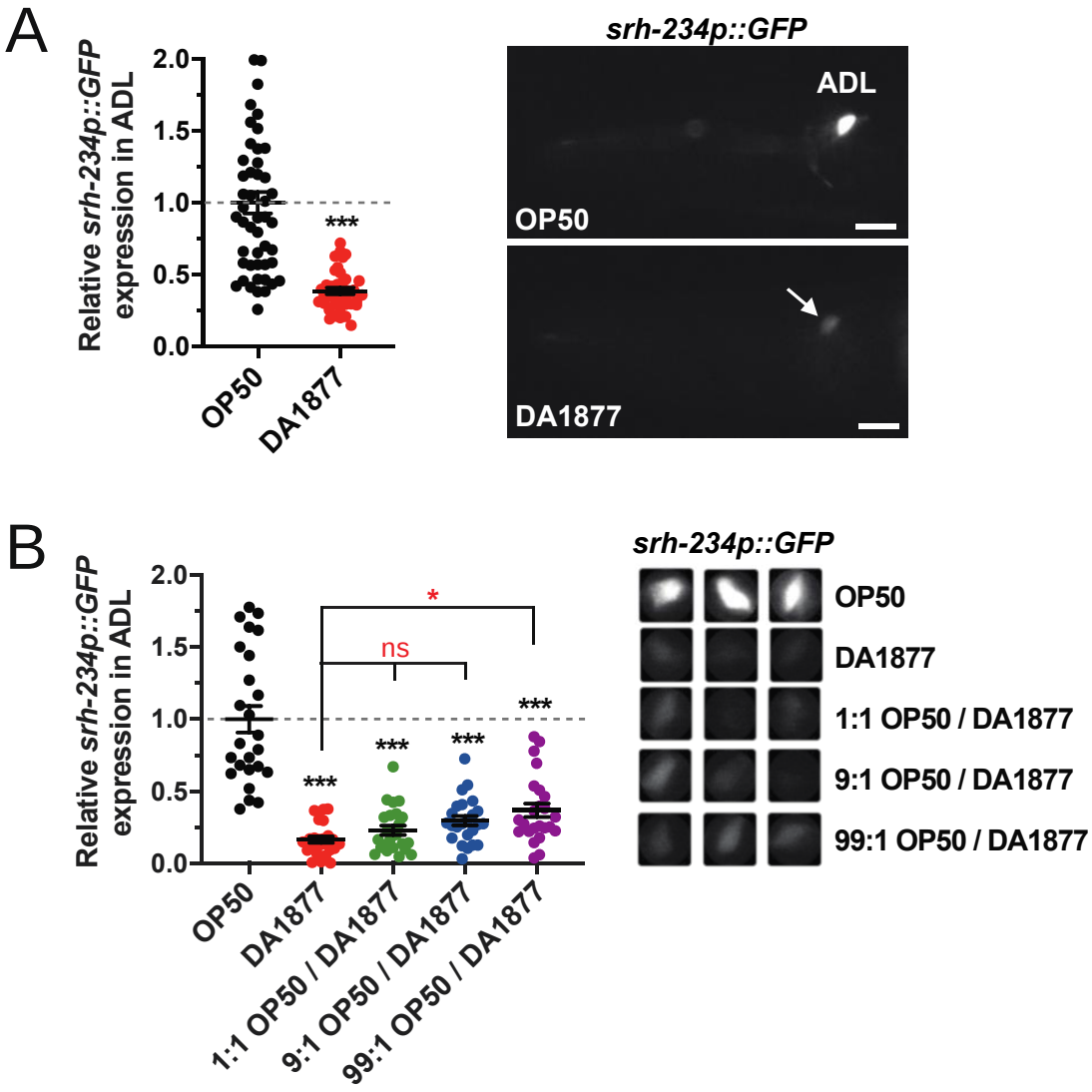


Figure 3. Expression of *srh-234* is repressed by a *Comamonas aq.* DA1877 diet.

(A) Relative expression levels of *srh-234* in the ADL cell body of adults fed either an *E. coli* OP50 diet or a *Comamonas aq.* DA1877 diet. Adult animals containing stably integrated copies of a *srh-234p::GFP* transgene (*oyIs56*) were examined at the same exposure time on both diets. Images are lateral views of the ADL sensory neuron. Scale is 15 μm. Data are represented as the mean ± SEM (n>38

animals for each diet). *** $p<0.001$ with an unpaired 2-tailed *t*-test. **(B)** Relative expression levels of *srh-234* in the ADL cell body of adult animals fed each of the indicated diets. 1:1, 9:1 and 99:1 refers to the dilution of *Comamonas aq.* DA1877 in *E. coli* OP50. Bacteria were seeded on peptone-free plates to prevent bacterial growth (see Material and Methods). Data are represented as the mean ± SEM (n>24 animals for each condition). The Kruskal-Wallis with Dunn multiple-comparisons test was used to determine the statistical significance of differences vs. wild-type animals fed *E. coli* OP50, with brackets indicating statistical differences between two specific diet conditions. ns, not significant, * $p<0.05$. Right panel: Representative cropped images of *srh-234p::GFP* expression in the ADL cell body of adults. Images were acquired at the same exposure time.

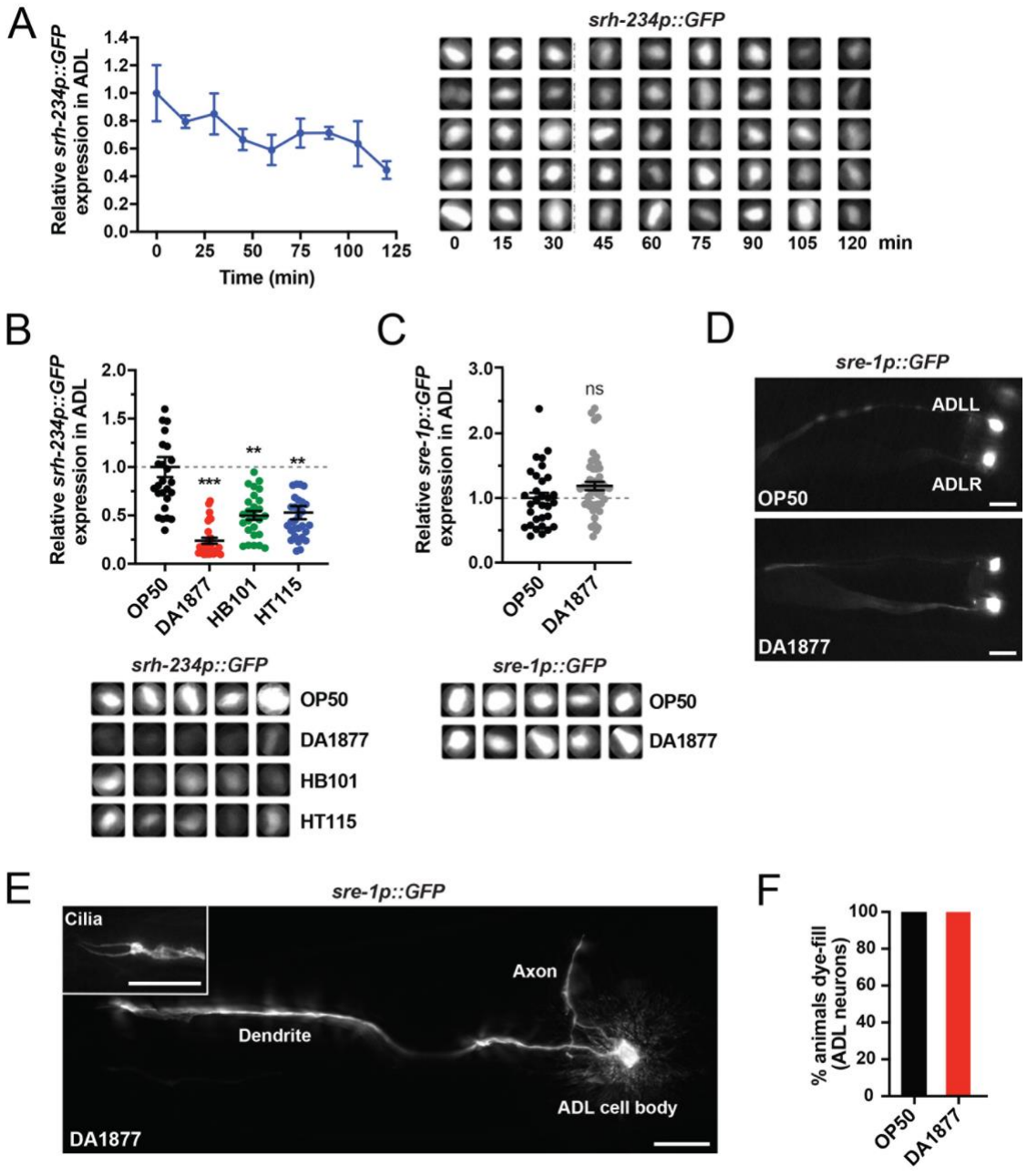


Figure 4. *srh-234* expression in ADL neurons is rapidly downregulated in the presence of *Comamonas aq.* DA1877.

(A) Time-course of the relative *srh-234p::GFP* expression levels in the ADL cell body of adult animals when transferred from an *E. coli* OP50 diet to a

Comamonas DA1877 diet. Expression of *srh-234p::GFP* in adults was measured every 15 min at the same exposure time. The graph shows the mean ± SEM (n=5 animals for each timepoint). Right panel: Representative cropped images of *srh-234p::GFP* expression in the ADL cell body in adult animals fed DA1877 at the indicated exposure times. **(B)** Relative expression levels of *srh-234p::GFP* in the ADL cell body of adults fed *E. coli* HB101 and HT115 diets compared to *E. coli* OP50 and *Comamonas aq.* DA1877 diets. Data are represented as the mean ± SEM (n>25 animals for each diet). The Kruskal-Wallis with Dunn multiple-comparisons test was used to determine the statistical significance of differences vs. wild-type animals fed *E. coli* OP50, ** $p<0.01$, *** $p<0.001$. Lower panel: Representative cropped images of *srh-234p::GFP* expression in the ADL cell body of adults fed different diets. **(C)** Relative expression of *sre-1p::GFP* in the ADL cell body of adult animals fed OP50 and DA1877 diets. Animals contain stably integrated copies of a *sre-1p::GFP* transgene (*otIs24*). Data are represented as the mean ± SEM (n>25 animals). ns, not significant by an unpaired 2-tailed *t*-test. Lower panel: Representative cropped images of *sre-1p::GFP* expression in the ADL cell body of adults fed OP50 and DA1877 diets. **(D)** Representative images of *sre-1p::GFP* expression of OP50- and DA1877-fed animals are a ventral view showing the left and right ADL neuron (anterior is left), and images were acquired at the same exposure time. Scale is 15 µm. **(E)** Representative z-stack image of *sre-1p::GFP* expression of DA1877-fed animals with ADL morphological structures labeled. An enlarged inset image of ADL cilia is shown. The image was captured using a Leica 3D Tissue Thunder microscope

with small volume computational clearing. Scale is 15 µm. **(F)** Percentage of adult animals with wild-type dye-filling of ADL neurons on OP50 and DA1877 diets. **(A-C)** Images were acquired at the same exposure time for comparison.

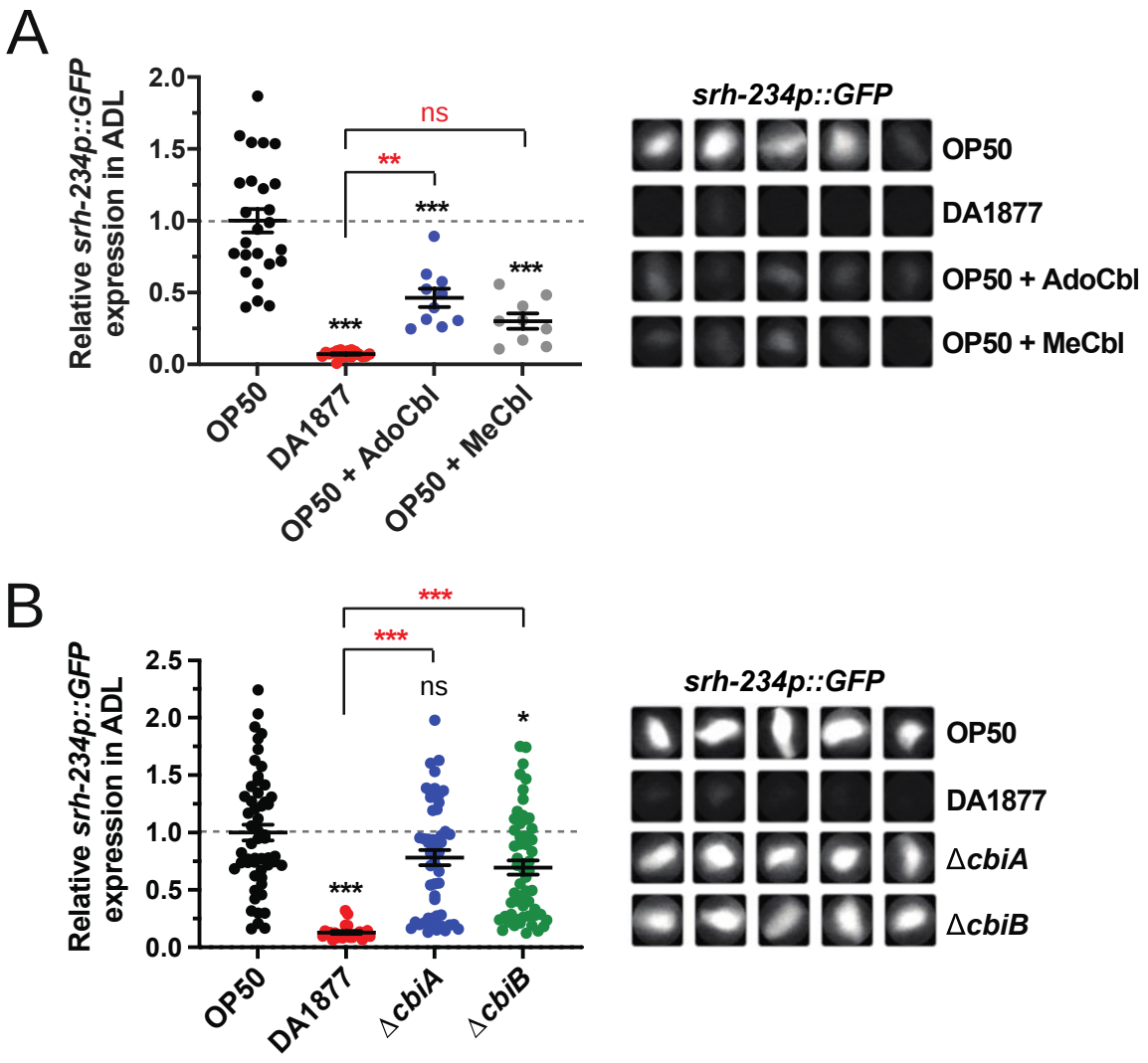


Figure 5. Vitamin B12 produced by *Comamonas aq.* DA1877 is required to repress *srh-234* expression.

(A) Relative expression of *srh-234* in the ADL cell body of OP50-fed adult animals supplemented with either Ado-Cbl or Me-Cbl compounds at a 64 nM final concentration. Data are represented as the mean ± SEM (n>9 animals for each condition). A one-way ANOVA with Tukey's multiple-comparisons test was used to determine the statistical significance of differences vs. wild-type animals fed *E. coli* OP50, with brackets indicating statistical differences between two specific

diet conditions. ns, not significant, ** $p<0.01$, *** $p<0.001$. **(B)** Relative expression of *srh-234* in the ADL cell body of adults fed the *Comamonas aq.* mutant strains Δ*cbiA* and Δ*cbiB* defective in producing vitamin B12 diets compared to *E. coli* OP50 and *Comamonas aq.* DA1877. Data are represented as the mean ± SEM (n=24-56 animals for each condition). The Kruskal-Wallis with Dunn multiple-comparisons test was used to determine the statistical significance of differences vs. wild-type animals fed *E. coli* OP50, with brackets indicating statistical differences between two specific diet condition. ns, not significant, *** $p<0.001$. **(A-B)** Right panels: Representative cropped images of *srh-234p::GFP* expression in the ADL cell body of adults. Images were acquired at the same exposure time for comparison.

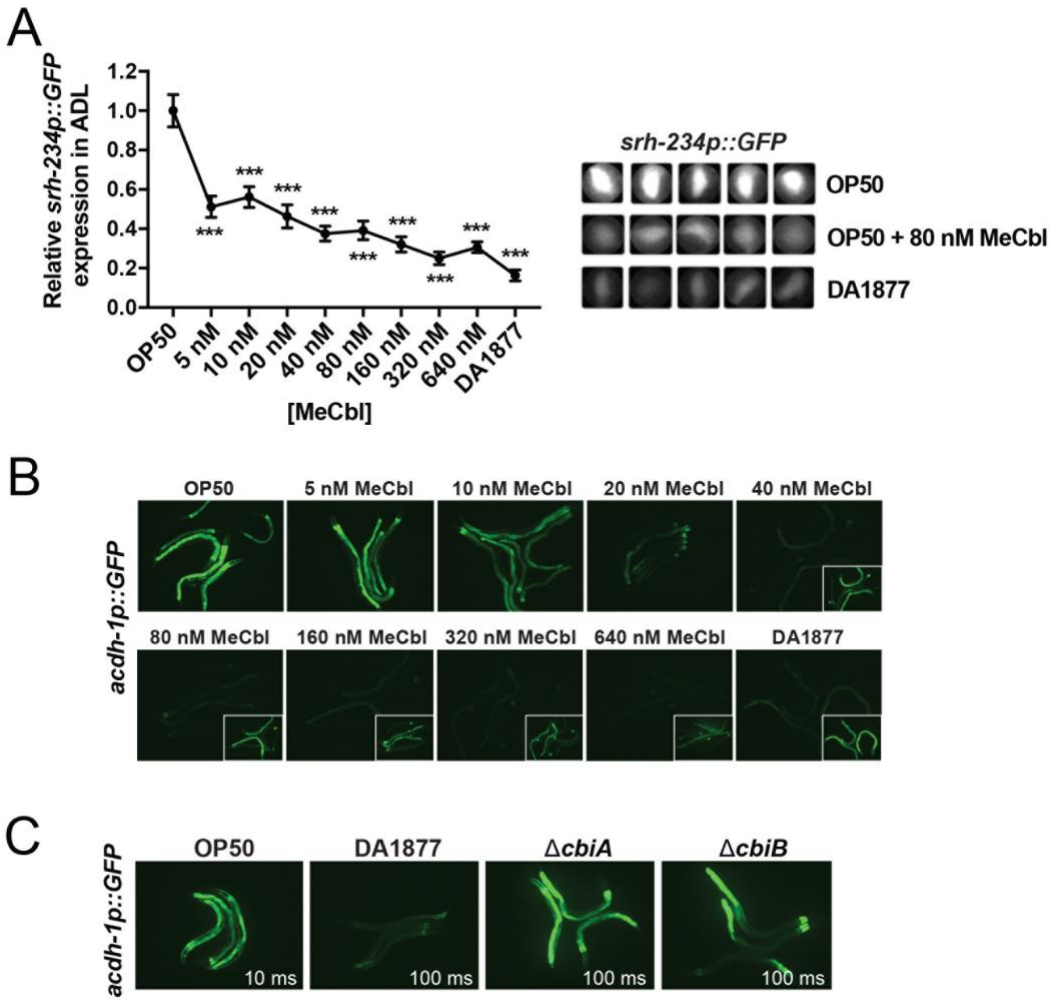


Figure 6. Dose dependent regulation of *srh-234* expression by vitamin B12. **(A-B)** Dose-dependent decrease in *srh-234p::GFP* expression in the ADL cell body **(A)** and *acdh-1p::GFP* expression in the intestine **(B)** of *E. coli* OP50-fed animals supplemented with increasing concentrations of Me-Cbl. The graph shows the mean ± SEM (n>12 animals for each time-point). A 2-way ANOVA with Tukey's multiple-comparisons test was used to determine the statistical significance of differences vs. wild-type animals fed *E. coli* OP50, *** $p<0.001$. Right Panel: Representative cropped images of *srh-234p::GFP* expression in the

ADL cell body acquired at the same exposure time. The dietary sensor *acdh-1p::GFP* was used as a positive control for the action of different Me-Cbl concentrations **(B)** and when animals were fed Δ*cbiA* and Δ*cbiA* mutants of *Comamonas aq.* **(C)** Representative images were acquired at the same exposure time for comparison unless indicated otherwise, and insets in panel B are images taken at a higher exposure time.

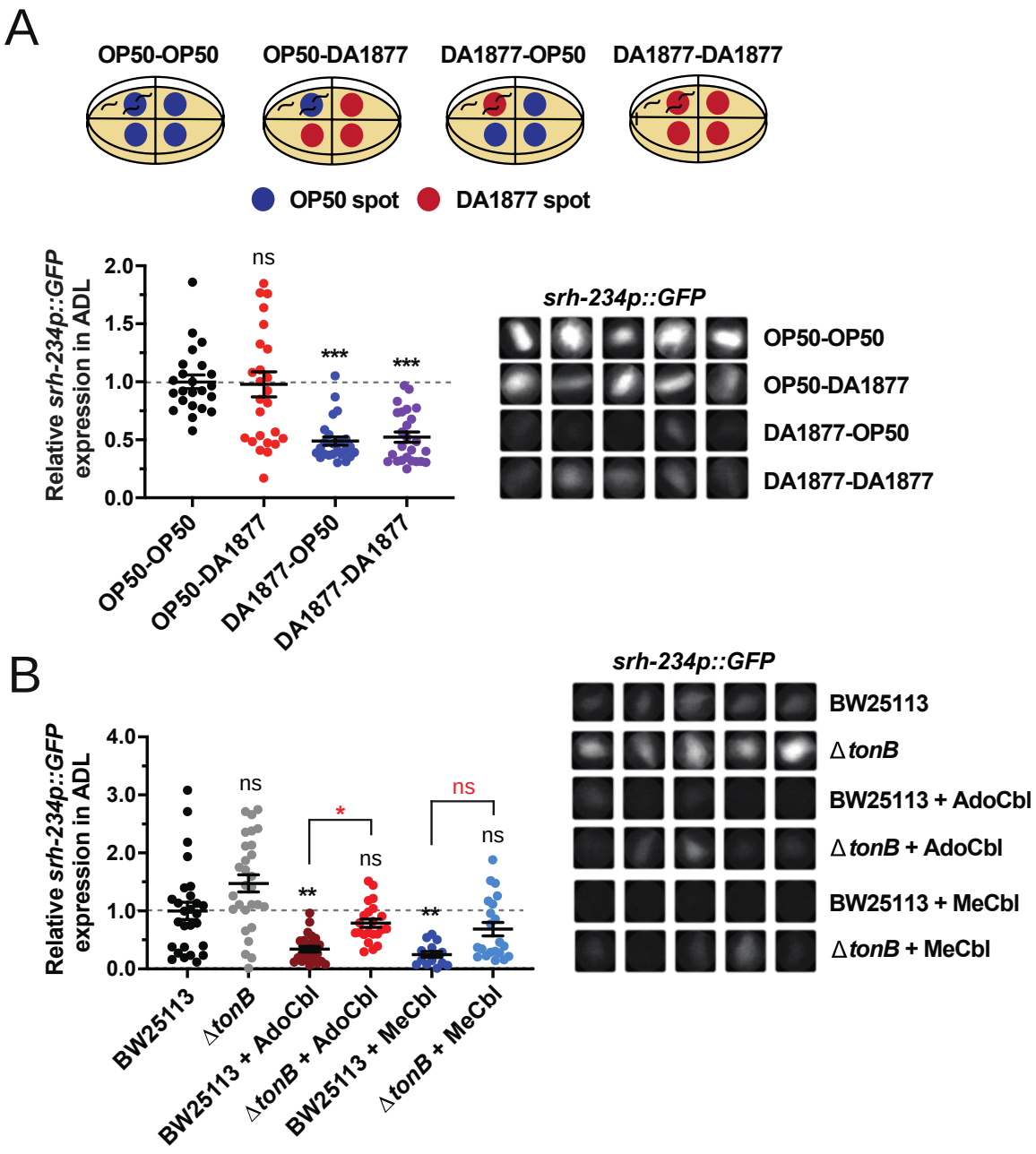


Figure 7. *E. coli* bacteria act as a vehicle for vitamin B12 uptake by *C. elegans*, to in turn regulate *srh-234* expression.

(A) Top panel: Schematic of a bacterial olfactory assay set-up using quadrant assay plates. Adult animals are placed into one quadrant allowing only one diet

to be ingested, while surrounding quadrants are seeded with either the *E. coli* OP50 diet (control) or the *Comamonas aq.* DA1877 diet. Lower panel: Relative expression of *srh-234p::GFP* in the ADL cell body of adults fed on either the *E. coli* OP50 or *Comamonas aq.* DA1877 diet on quadrant plates with surrounding inaccessible diets. Data are represented as the mean ± SEM (n>23 animals for each diet). The Kruskal-Wallis with Dunn multiple-comparisons test was used to determine the statistical significance of differences vs. wild-type animals fed *E. coli* (OP50-OP50), with brackets indicating statistical differences between two specific conditions. ns, not significant, *** $p<0.001$. **(B)** Relative expression of *srh-234* in the ADL cell body of adults fed the *E. coli* Δ*tonB* mutant (strain JW5195) compared to its parental wild-type strain (BW25113) supplemented with or without Ado-Cbl and Me-Cbl (64 nm final concentrations). Data are represented as the mean ± SEM (n=14-28 animals for each condition). The Kruskal-Wallis with Dunn multiple-comparisons test was used to determine the statistical significance of differences vs. wild-type animals fed *E. coli* BW25113, with brackets indicating statistical differences between two specific bacterial genotypes and conditions. ns, not significant, * $p<0.05$, ** $p<0.01$. Right panel: *srh-234p::GFP* is weakly expressed in adults when fed *E. coli* BW25113 compared to *E. coli* OP50. **(A-B)** Representative cropped images of *srh-234p::GFP* expression in the ADL cell body of adults. Images were acquired at the same exposure time for comparison.

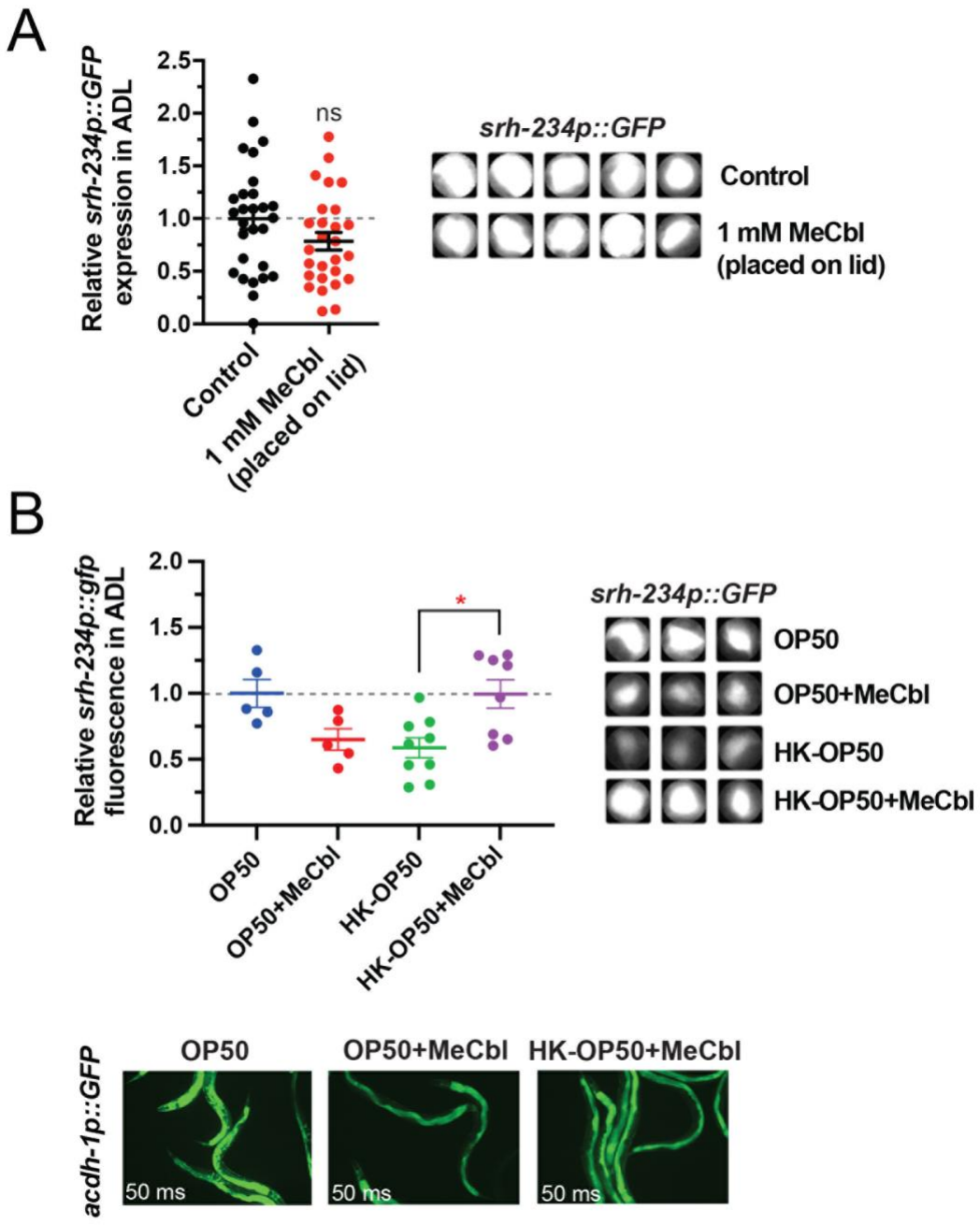


Figure 8. Vitamin B12 does not act as a volatile cue to alter *srh-234* expression levels in ADL sensory neurons.

(A) Relative expression of *srh-234p::GFP* in the ADL cell body of adult animals fed *E. coli* OP50 in the presence 1 mM Me-Cbl placed on the cover of the petri

dish lid. Data are represented as the mean ± SEM (n>25 animals). ns, not significant by an unpaired 2-tailed *t*-test. Right panel: Representative cropped images of *srh-234p::GFP* expression in the ADL cell body of adults. Images were acquired at the same exposure time. **(B)** Relative expression of *srh-234* in the ADL cell body of adults that are fed 48 hours with either live or heat-killed *E. coli* OP50 supplemented with vitamin B12 (Me-Cbl, 64 nM). Data are represented as the mean ± SEM. The Kruskal-Wallis with Dunn multiple comparisons test was used to determine the statistical significance of differences vs. wild-type animals fed live *E. coli* OP50, with brackets indicating the statistical differences between two specific conditions. * $p<0.05$**.** Right panel: Representative cropped images of *srh-234p::GFP* expression in the ADL cell body of adults. Images were acquired at the same exposure time. Bottom panel: Representative images of *acdh-1p::GFP* expression in adult animals at the indicated exposure times.

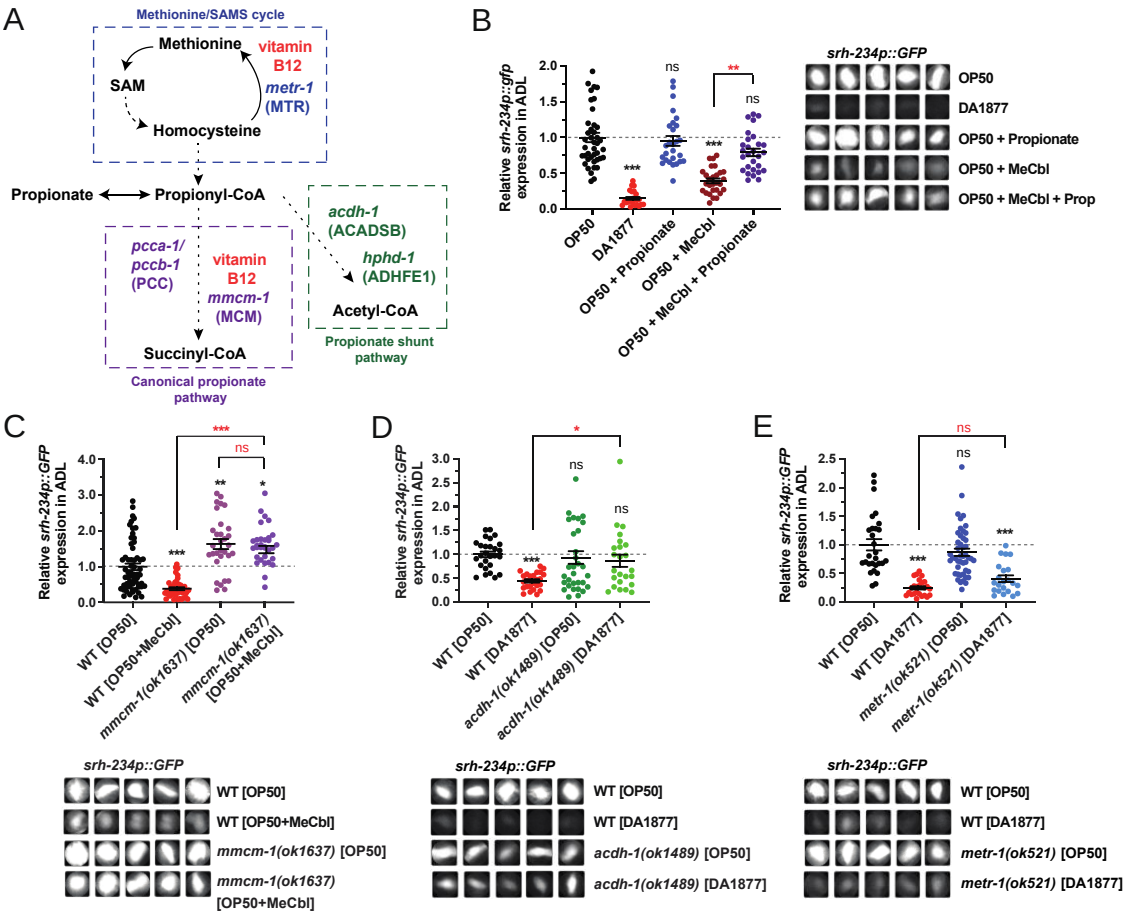


Figure 9. The repressive effects of vitamin B12 on *srh-234* expression is reversed by propionate accumulation.

(A) Schematic of the *C. elegans* vitamin B12-dependent methionine/SAMs cycle (blue dotted box), the vitamin B12-dependent canonical propionate breakdown pathway (purple dotted box), and the propionate shunt pathway independent of vitamin B12 (green dotted box). Genes shown are: *metr-1*/MTR (methionine synthase), *pcca-1*/PCCA and *pccb-1*/PCCB (two members of the propionyl-CoA carboxylase complex), *mmcm-1*/MCM (methylmalonyl-CoA mutase), *acdh-1*/ACADSB (acyl-CoA dehydrogenase) and *hphd-1*/ADHFE1 (3-hydroxypropionate-oxoacid transhydrogenase). **(B)** Relative expression of *srh-*

234 in the ADL cell body of adults fed an *E. coli* OP50 diet supplemented with excess propionate (40 mM final concentration) and/or Me-Cbl (64 nM final concentration). Data are represented as the mean ± SEM (n=24-42 animals for each condition). A one-way ANOVA with Tukey's multiple-comparisons test was used to determine the statistical significance of differences vs. wild-type animals fed *E. coli* OP50, with brackets indicating statistical differences between two specific diet conditions. ns, not significant, ** $p<0.01$, *** $p<0.001$. **(C-E)** Relative expression of *srh-234* in the ADL cell body of adult animals with mutations in the canonical propionate breakdown pathway **(C)**, the propionate shunt breakdown pathway **(D)**, and the methionine/SAM cycle **(E)** fed *E. coli* OP50 with or without exogenous vitamin B12 (Me-Cbl, 64 nM), or *Comamonas aq.* DA1877. Data are represented as the mean ± SEM. n>18 animals for each diet and genotype. **(C-E)** The Kruskal-Wallis with Dunn multiple-comparisons test was used to determine the statistical significance of differences vs. wild-type animals fed *E. coli* OP50, with brackets indicating statistical differences between two specific genotypes and conditions. ns, not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. **(B-E)** Representative cropped images of *srh-234p::GFP* expression in the ADL cell body of adults. Images were acquired at the same exposure time for comparison.

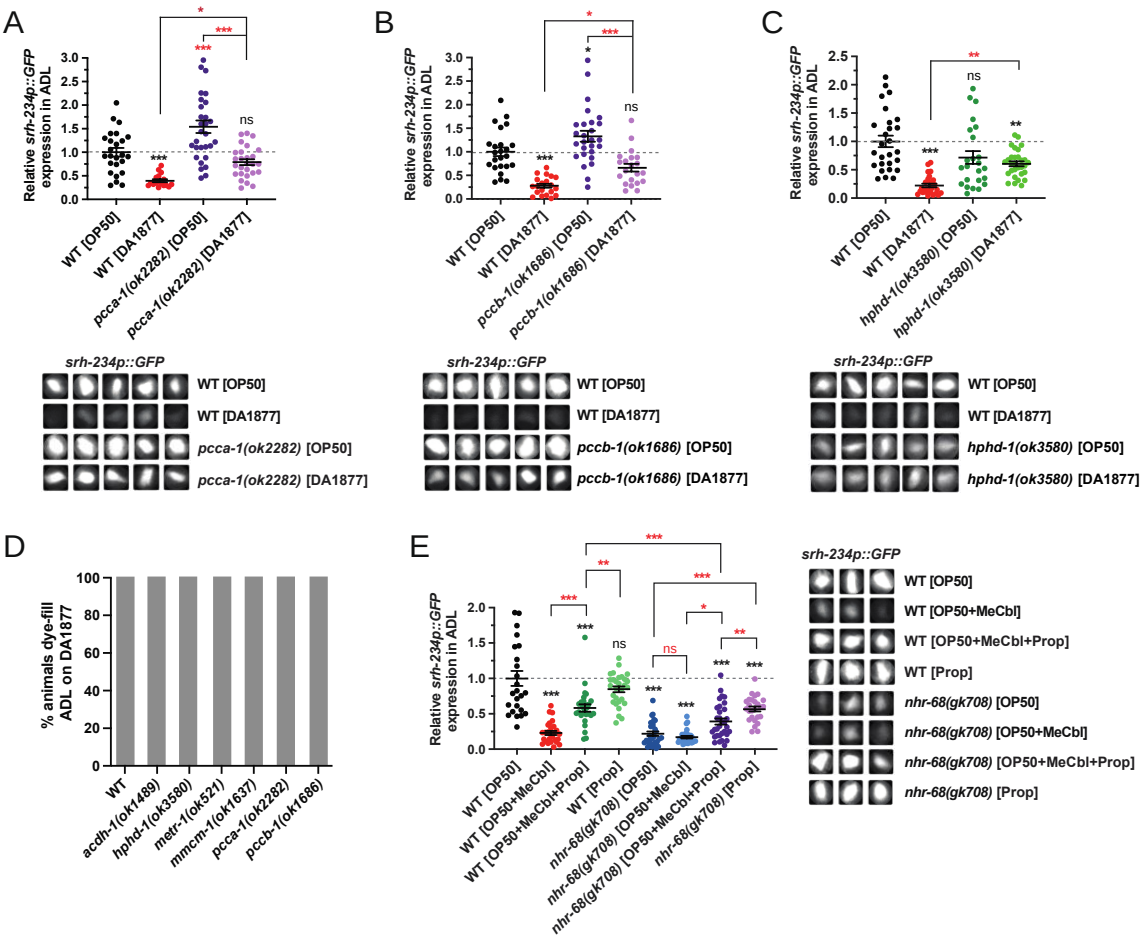


Figure 10. Vitamin B12 decrease *srh-234* expression but not in animals with mutations in the *pccb-1*, *pcca-1,* and *hphd-1* genes.

(A-C) Relative expression of *srh-234p::GFP* in the ADL cell body of adult animals with mutations in the canonical propionate breakdown pathway genes, *pcca-1,* and *pccb-1* (propionyl-CoA carboxylase) **(A-B)** and the propionate shunt gene *hdpd-1* (3-hydroxypropionate-oxoacid transhydrogenase) **(C)** when fed either *E. coli* OP50 or *Comamonas aq.* DA1877 diets. Lower panels: Representative cropped images of *srh-234p::GFP* expression in the ADL cell body. **(D)** Percentage of adult animals showing wild-type dye-filling of ADL neurons when fed a *Comamonas aq.* DA1877 diet for the indicated mutant genotypes. **(E)**

Relative expression of *srh-234p::GFP* in the ADL cell body of *nhr-68* mutants fed *E. coli* OP50 supplemented with or without 64nM Me-Cbl and/or 40mM propionate. **(A-D)** Representative images were acquired at the same exposure time for comparison. Data are represented as the mean ± SEM (n>20 animals). The Kruskal-Wallis with Dunn multiple-comparisons test was used to determine the statistical significance of differences vs. wild-type animals fed *E. coli* OP50, with brackets indicating statistical differences between two specific conditions and genotypes. ns, not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Right panel: Representative cropped images of *srh-234p::GFP* expression in the ADL cell body. **(A-C, E)** Images were acquired at the same exposure time for comparison.

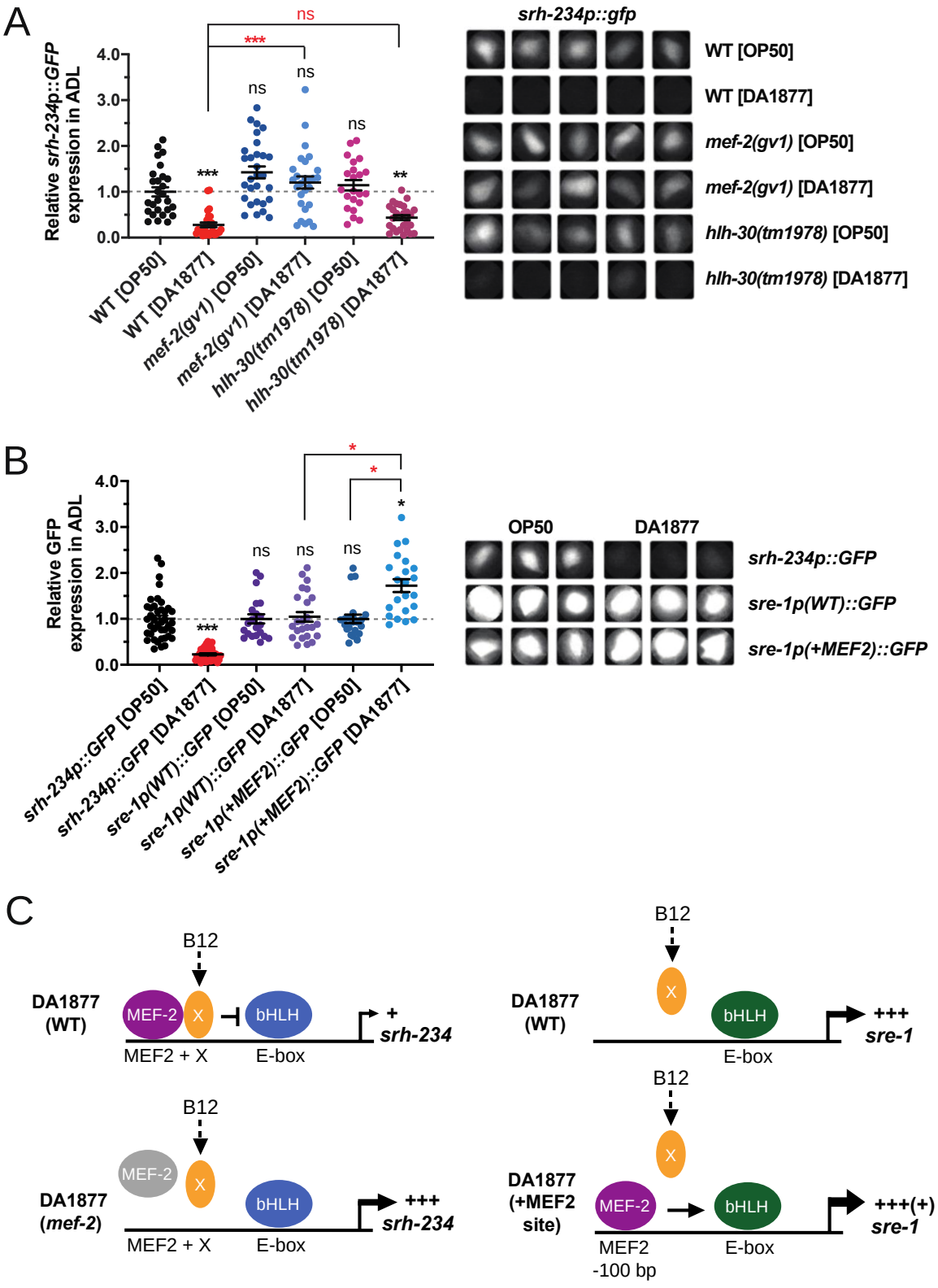

A
Relative srh-234p::GFP expression in ADL
4.0
3.0
2.0
1.0
0.0
ns
***
ns
ns
ns
***
**
WT [OP50]
WT [DA1877]
mef-2(gv1) [OP50]
mef-2(gv1) [DA1877]
hlh-30(tm1978) [OP50]
hlh-30(tm1978) [DA1877]
srh-234p::gfp
WT [OP50]
WT [DA1877]
mef-2(gv1) [OP50]
mef-2(gv1) [DA1877]
hlh-30(tm1978) [OP50]
hlh-30(tm1978) [DA1877]
B
Relative GFP expression in ADL
4.0
3.0
2.0
1.0
0.0
*
*
*
***
ns
ns
ns
srh-234p::GFP [OP50]
srh-234p::GFP [DA1877]
sre-1p(WT)::GFP [OP50]
sre-1p(WT)::GFP [DA1877]
sre-1p(+MEF2)::GFP [OP50]
sre-1p(+MEF2)::GFP [DA1877]
OP50
DA1877
srh-234p::GFP
sre-1p(WT)::GFP
sre-1p(+MEF2)::GFP
C
B12
DA1877 (WT)
MEF-2
X
bHLH
+ srh-234
MEF2 + X
E-box
B12
DA1877 (mef-2)
MEF-2
X
bHLH
+++ srh-234
MEF2 + X
E-box
B12
X
DA1877 (WT)
bHLH
+++ sre-1
E-box
B12
X
DA1877 (+MEF2 site)
MEF-2
bHLH
+++(+) sre-1
MEF2 -100 bp
E-box

Figure 11. *mef-2* is required for vitamin B12-dependent regulation of *srh-234.*

(A) Relative expression of *srh-234* in the ADL cell body of *mef-2* and *hlh-30* mutants when adults are fed a *Comamonas aq.* DA1877 diet compared to an *E. coli* OP50 diet. Data are represented as the mean ± SEM (n>22 animals). The Kruskal-Wallis with Dunn multiple-comparisons test was used to determine the statistical significance of differences vs. wild-type animals fed *E. coli* OP50, with brackets indicating statistical differences between two specific genotypes and conditions. ns, not significant, ** $p<0.01$, *** $p<0.001$. Right panel: Representative cropped images of *srh-234p::GFP* expression in the ADL cell body. Images were acquired at the same but at a low exposure time. **(B)** Relative expression of wild-type *sre-1p::GFP* (*sre-1p(WT)::GFP*) or the *sre-1* promoter with the inserted MEF2 binding site sequence from the *srh-234* promoter (*sre-1p(+MEF2)::GFP*) in the ADL cell body of adults fed a *Comamonas aq.* DA1877 diet compared to an *E. coli* OP50 diet. Data are represented as the mean ± SEM (n=21-40 animals). For *sre-1* expression, data were normalized to the *sre-1p::GFP* reporter for animals fed *E. coli* OP50. For *srh-234* expression, data were normalized to *srh-234p::GFP* for animals fed *E. coli* OP50. The Kruskal-Wallis with Dunn multiple-comparisons test was used to determine the statistical significance of differences vs. wild-type animals fed *E. coli* OP50, with brackets indicating statistical differences between two specific genotypes and conditions. ns, not significant, * $p<0.05$, *** $p<0.001$. Right panel: Representative cropped images of GFP expression under control of the *srh-234* or *sre-1* promoter with or

without the *srh-234* MEF-2 binding site in the ADL cell body. Images were acquired at the same exposure time. **(C)** Model based on findings shown in panel B explaining the observed expression changes for *srh-234* in *mef-2* mutants, and *sre-1* with a *srh-234* MEF2-binding site artificially inserted in its promoter upstream and close to the identified E-box that drives *sre-1* expression in the ADL neuron. +, +++, and +++(+) indicates low, high, and highly increased expression levels, respectively.

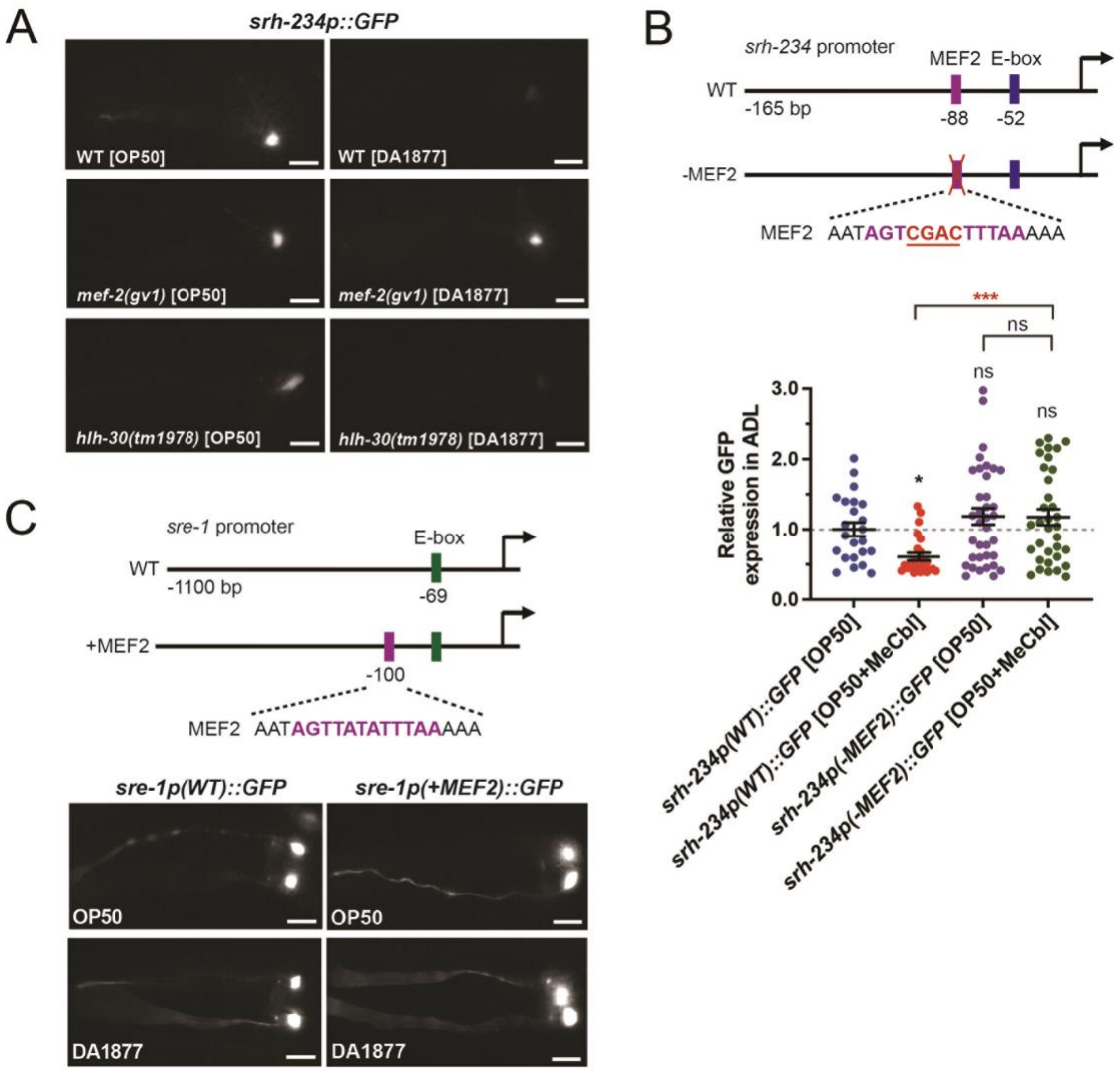


Figure 12. Introduction of the *srh-234* MEF2 site in the *cis*-regulatory region of *sre-1* does not confer regulation by vitamin B12.

(A) Representative images of *srh-234p::GFP* expression in the ADL cell body of *mef-2* and *hlh-30* mutants fed on *Comamonas aq.* DA1877 compared to *E. coli* OP50 diets. Images were taken at the same exposure time (anterior is left). Scale is 15 µm. **(B)** Upper panel: The indicated lengths and positions of predicted regulatory elements relative to the translational start site of *srh-234* fused to the

gfp coding sequence in an expression vector. Sequences in blue and purple indicate the predicted E-box motif and the mutated sequence in the core MEF2 binding site of *srh-234* (AGTTATATTTAA to AGTCGACTTTAA), respectively. Lower panel: Relative expression of *srh-234* in ADL driven by the wild-type *srh-234* promoter sequence (*srh-234p(WT)::GFP*) or with the mutated MEF2 binding site (*srh-234p(-MEF2)::GFP*) in adult animals fed *E. coli* OP50 supplemented with or without vitamin B12 (64 nM Me-Cbl final concentration). **(C)** Upper panel: The indicated lengths and positions of predicted regulatory elements relative to the translational start site of *sre-1* fused to the *gfp* coding sequence in an expression vector. Sequences in purple and green indicate the predicted E-box motif of *sre-1* with the artificially inserted MEF2 binding site sequence of *srh-234* near the *sre-1* E-box site, respectively. Lower panel: Representative expression of *sre-1* in ADL driven by the wild-type *sre-1* promoter sequence without (*sre-1p(WT)::GFP*) or with the *srh-234* MEF2 binding site inserted (*sre-1p(+MEF2)::GFP*) in adult animals fed on *Comamonas aq.* DA1877 compared to *E. coli* OP50. Images show both left and right cell bodies of ADL neurons (anterior is left) and were acquired at the same exposure time. Scale is 15 µm.

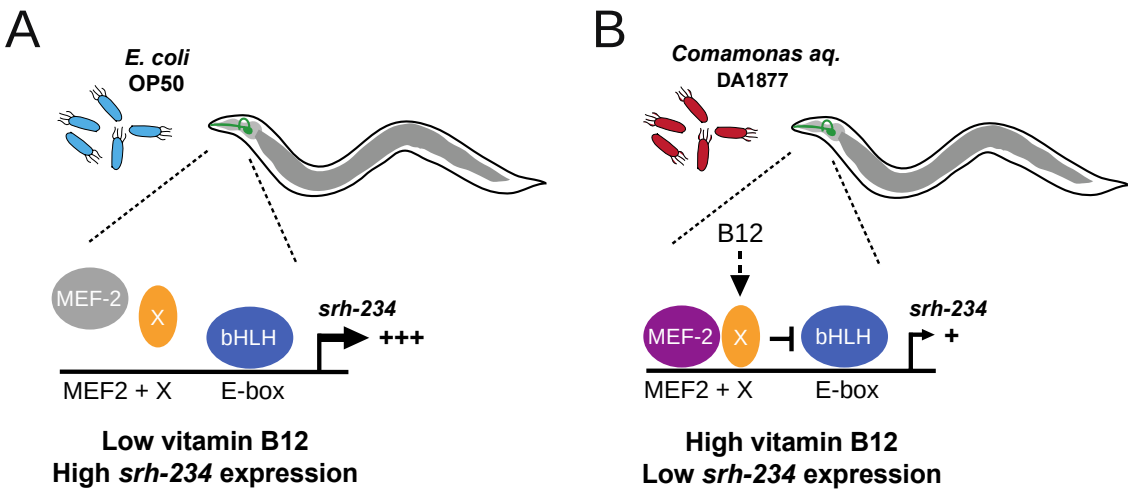
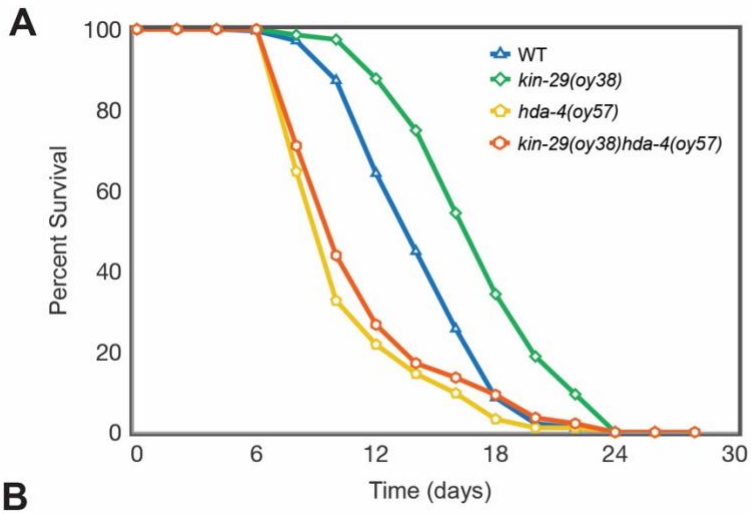


Figure 13. Model for the regulation of *srh-234* chemoreceptor expression levels in the ADL sensory neuron under different dietary conditions.

Expression levels of *srh-234* in *C. elegans* animals is high (+++) when fed a low vitamin B12 diet of *E. coli* OP50 bacteria **(A)** but low (+) when fed a high vitamin B12 diet of *Comamonas aq.* DA1877 bacteria **(B)**. An unknown factor (X) may act together with the MEF-2 transcription factor to repress *srh-234* expression levels under conditions of high vitamin B12 via a bHLH/E-box module important to promote expression of *srh-234* in ADL.



B

Genotype	Strain number	# Rep.	Mean lifespan ± SEM (days)	# deaths/ total number of animals	*p*-value against WT, Log-Rank (Mantel-cox)	*p*-value against *hda-4*, Log-Rank (Mantel-cox)
WT	N2	1	14.2 ± 0.4	82/101	n/a	n/a
kin-29(oy38)	PY1476	1	18.2 ± 0.4	50/100	<0.0001	n/a
kin-29(oy38)hda-4(oy57)	PY4112	1	10.0 ± 0.7	53/100	<0.0001	n/a
WT	N2	2	14.9 ± 0.4	59/147	n/a	<0.0001
kin-29(oy38)	PY1476	2	17.3 ± 0.6	37/152	0.0008	<0.0001
hda-4(oy57)	PY4111	2	10.4 ± 0.4	74/157	<0.0001	n/a
kin-29(oy38)hda-4(oy57)	PY4112	2	11.7 ± 0.5	89/151	<0.0001	0.0270
WT	N2	3	14.3 ± 0.4	56/152	n/a	<0.0001
kin-29(oy38)	PY1476	3	17.6 ± 0.4	70/151	<0.0001	<0.0001
hda-4(oy57)	PY4111	3*	7.3 ± 0.3	107/151	<0.0001	n/a
kin-29(oy38)hda-4(oy57)	PY4112	3*	7.8 ± 0.3	98/150	<0.0001	0.1202
WT	N2	4	14.6 ± 0.3	115/299	n/a	<0.0001
kin-29(oy38)	PY1476	4	17.5 ± 0.4	108/303	<0.0001	<0.0001
hda-4(oy57)	PY4111	4	11.0 ± 0.3	181/308	<0.0001	n/a
kin-29(oy38)hda-4(oy57)	PY4112	4	11.8 ± 0.3	186/300	<0.0001	0.0323

Figure 14. Mutations in *hda-4* suppress the extended lifespan of *kin-29* mutants.

Lifespan was determined by assaying the percent survival of animals as a function of time. Scoring was performed every other day on NGM plates containing 5 μM FUDR and concentrated *E. coli* OP50 bacteria (see methods). Mean lifespans were with n>100 animals for each genotype (days ± SEM): wild-type, *kin-29(oy38)* and *hda-4(oy57)* single mutants, and *kin-29(oy38)hda-4(oy57)*

double mutants. **(A)** Data is shown from a single biological repeat (#4) with all strains examined in parallel. The mean lifespan of *hda-4* single and *kin-29hda-4* double mutants is significantly different from that of wild-type and *kin-29* single mutants, whereas the mean lifespan of *hda-4* single mutants is not statistically different from *kin-29hda-4* double mutants (log-rank test, $p<0.01$). * indicates animals fed at a higher food density. **(B)** Data of each biological repeat and a summary of lifespan statistics.

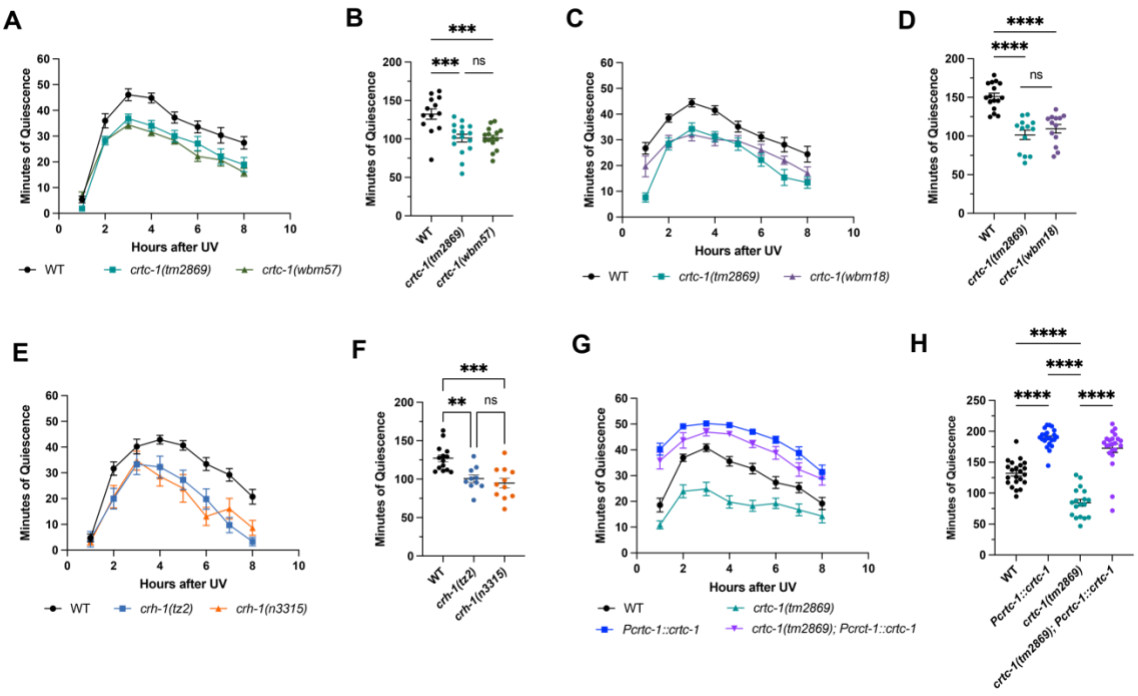


Figure 15. *crtc-1* and *crh-1* mutants have reduced movement quiescence.

(A) Time course of movement quiescence per hour over 8 hours for wild-type, *crtc-1(tm2869)* loss-of-function mutants, and *crtc-1(wbm57)* null mutant animals (*n* ≥ 12 animals for each genotype) after UVC irradiation (1,500 J/m^2). **(B)** Minutes of quiescence during the first 4 hours after UVC exposure for wild-type, *crtc-1(tm2869)* loss-of-function mutants, and *crtc-1(wbm57)* null mutant animals (*n* ≥ 12 animals for each genotype. **(C)** Time course of movement quiescence per hour over 8 hours for wild-type, *crtc-1(tm2869)* loss-of-function mutants, and *crtc-1(wbm18)* CBD mutant animals (*n* ≥ 12 animals for each genotype) after UVC irradiation (1,500 J/m^2). **(D)** Minutes of quiescence during the first 4 hours after UVC exposure for wild-type, *crtc-1(tm2869)* loss-of-function mutants, and *crtc-1(wbm18)* CBD mutant animals (*n* ≥ 12 animals for each genotype. **(E)** Time course of movement quiescence per hour over 8 hours for wild-type, *crh-1(tz2)*

and *crh-1(n3315)* loss-of-function mutants (*n* ≥ 8 animals for each genotype) after UVC irradiation (1,500 J/m^2). **(F)** Minutes of quiescence during the first 4 hours after UVC exposure for wild-type, *crh-1(tz2)* and *crh-1(n3315)* loss-of-function mutants (*n* ≥ 8 animals for each genotype. **(G)** Time course of movement quiescence per hour over 8 hours for wild-type, *crtc-1(tm2869)* loss-of-function mutants*, Pcrtc-1::crtc-1, crtc-1(tm2869);* and *Pcrtc-1::crtc-1* animals (*n* > 20 animals for each genotype) after UVC irradiation (1,500 J/m^2). **(H)** Minutes of quiescence during the first 4 hours after UVC exposure for wild-type, wild-type, *crtc-1(tm2869)* loss-of-function mutants*, Pcrtc-1::crtc-1, crtc-1(tm2869);* and *Pcrtc-1::crtc-1* animals (*n* > 20 animals for each genotype). **(A-H)** Data are represented as the mean ± SEM. ****, ***, **, ns indicate corrected *p*-values at $p < 0.0001$, $p < 0.001$, $p < 0.01$ and not significant, respectively, by ordinary one-way ANOVA with Tukey's multiple-comparisons test.

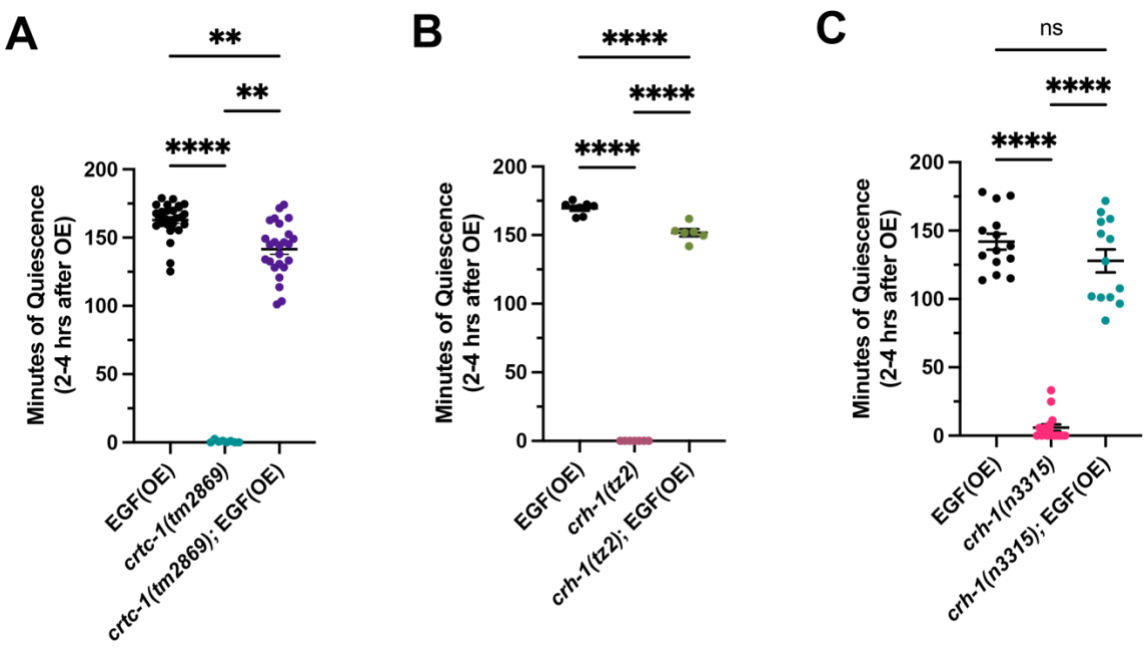


Figure 16. *crtc-1* and *crh-1* act upstream of EGF and sleep promoting neurons ALA and RIS to regulate sleep.

To induce EGF OE, **(A)** *crtc-1* and **(B and C)** two alleles of *crh-1* mutant adult animals expressing a *Phsp-16.2*::*LIN-3C* transgene were heat-shocked in a 33°C circulating water bath for 30 min. Minutes of quiescence were measured between hours 2-4 following heat-shock. Data are represented as mean ± SEM. ****, ** indicate corrected *p*-values that are different from wild-type or mutants at $p < 0.0001$ and $p < 0.01$ respectively by **(B)** an ordinary one-way ANOVA with Tukey's multiple comparisons test or **(A and C)** a Kruskal-Wallis test with Dunn multiple-comparisons test.

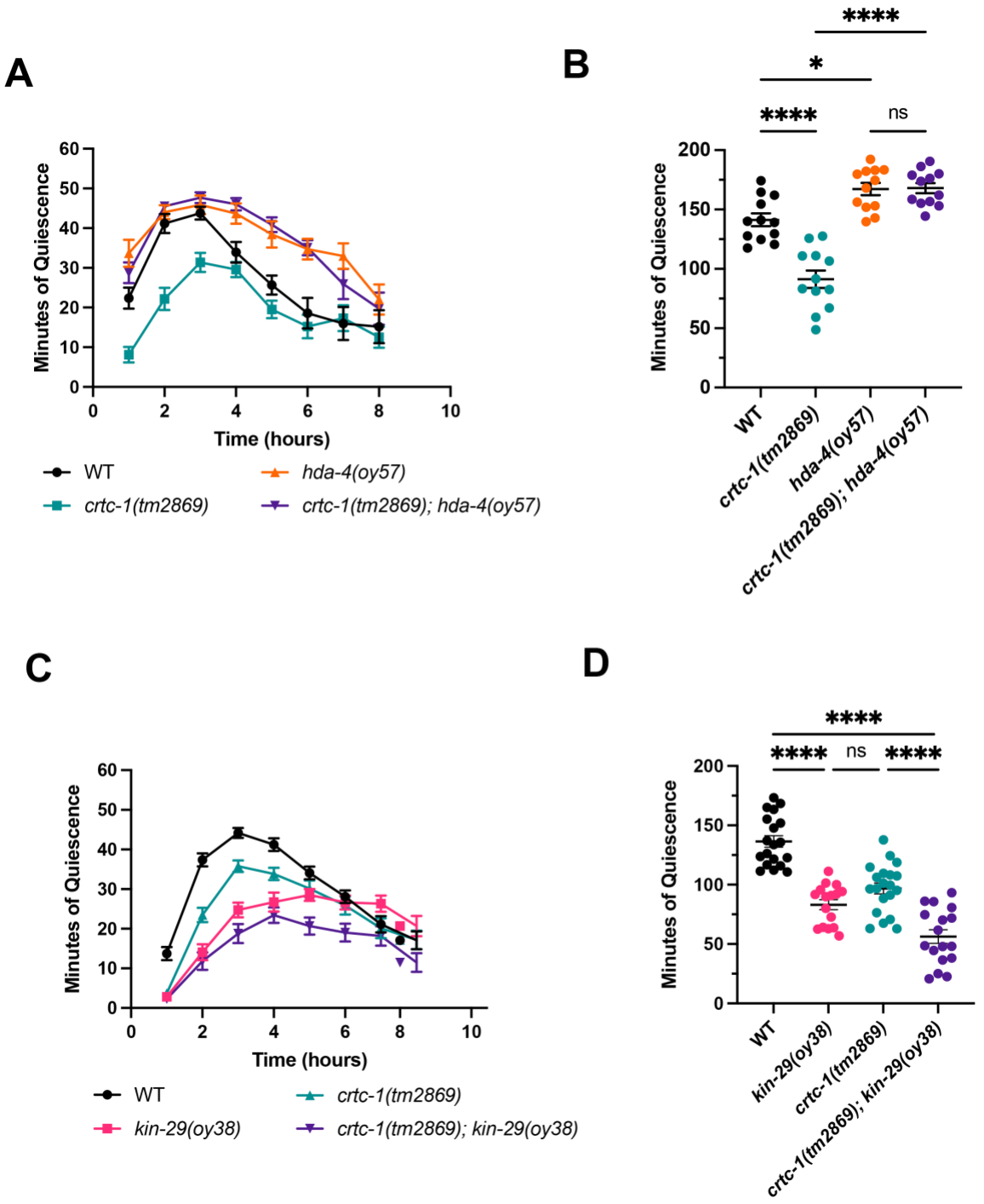


Figure 17. *hda-4* and *crtc-1* may act downstream of KIN-29/SIK to regulate sleep.

(A) Time course of movement quiescence per hour over 8 hours for wild-type, *crtc-1, hda-4* single, and *crtc-1; hda-4* double mutant animals (*n* = 12 animals for each genotype) after UVC irradiation (1,500 J/m^2). **(B)** Total minutes of quiescence during the first 4 hours after UVC exposure for wild-type, *crtc-1, hda-4* single, and *crtc-1; hda-4* double mutant animals (*n* = 12 animals for each genotype). **(C)** Time course of movement quiescence per hour over 8 hours for wild-type, *kin-29*, *crtc-1* single, and *crtc-1; kin-29* double mutant animals (*n* = 16-20 animals for each genotype) after UVC irradiation (1,500 J/m^2). **(D)** Minutes of quiescence during the first 4 hours after UVC exposure for wild-type, *kin-29*, *crtc-1* single, and *crtc-1; kin-29* double mutant animals (*n* = 16-20 animals for each genotype). **(E)** Feeding quiescence of wild-type, *kin-29*, *crtc-1* single, and *crtc-1; kin-29* double mutant animals (n > 20 animals for each genotype) measured 2 hours after UVC irradiation (1,500 J/m2). **(B-D)** Data are represented as the mean ± SEM. ****, *, ns indicate corrected *p*-values at $p < 0.0001$, $p < 0.05$ and not significant, respectively, by ordinary one-way ANOVA with Tukey's multiple-comparisons test.

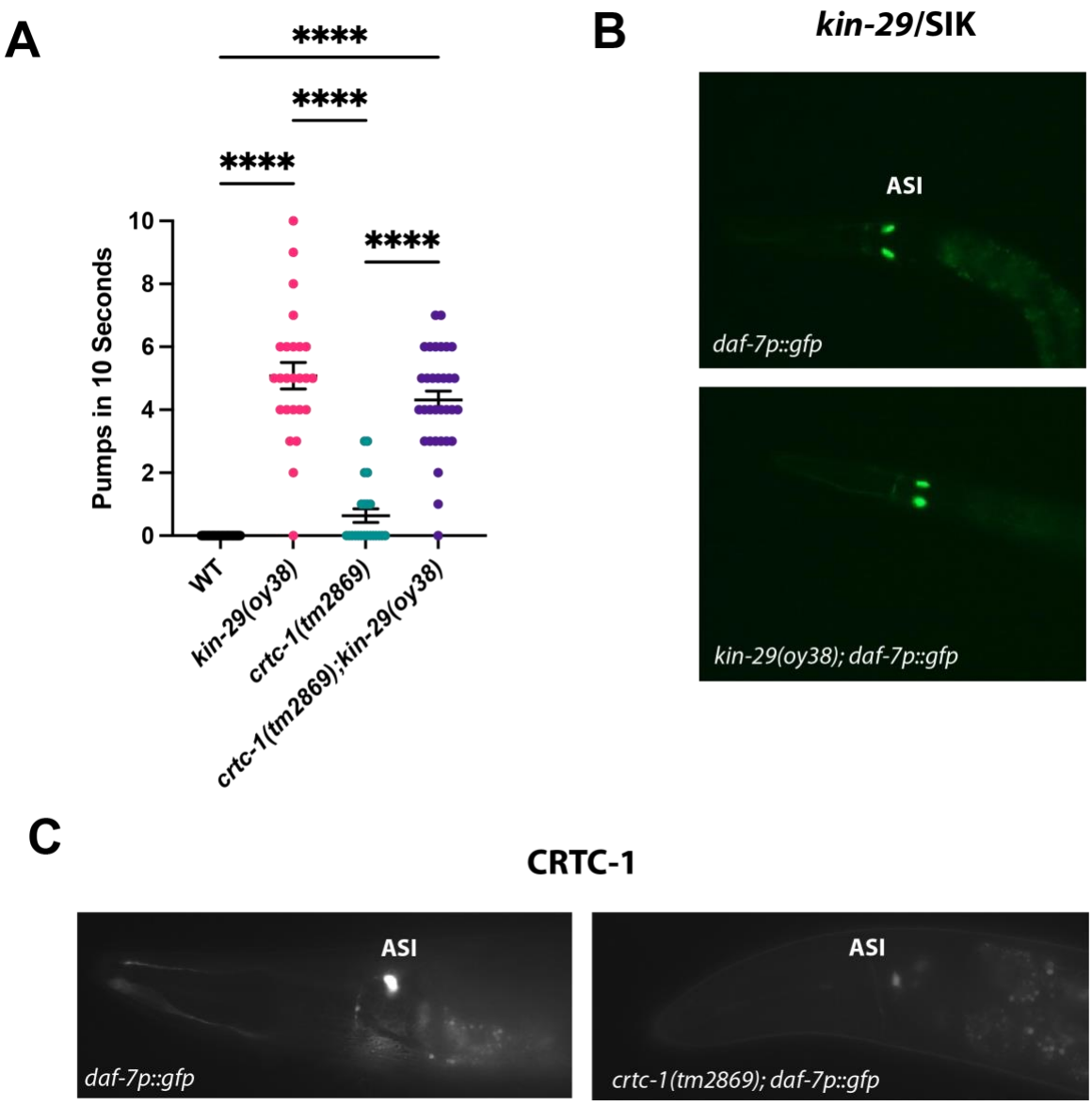


Figure 18: *crtc-1* may act with DAF-7/TGF-β in the food-dependent plasticity pathway of SIS.

(A) Feeding quiescence of wild-type, *kin-29*, *crtc-1* single, and *crtc-1; kin-29* double mutant animals (n > 20 animals for each genotype) measured 2 hours after UVC irradiation (1,500 J/m2). Data are represented as the mean ± SEM. **** indicates corrected *p*-values at $p < 0.0001$ by ordinary one-way ANOVA with

Tukey's multiple-comparisons test. **(B-C)** Representative images of *daf-7p::gfp* expression in the ASI neurons. **(B)** Mutations in *kin-29* do not affect *daf-7p::gfp* expression as found by (Lanjuin & Sengupta, 2002) and verified here. **(C)** Mutations in *crtc-1* regulate *daf-7p::gfp* expression in ASI neurons**.**

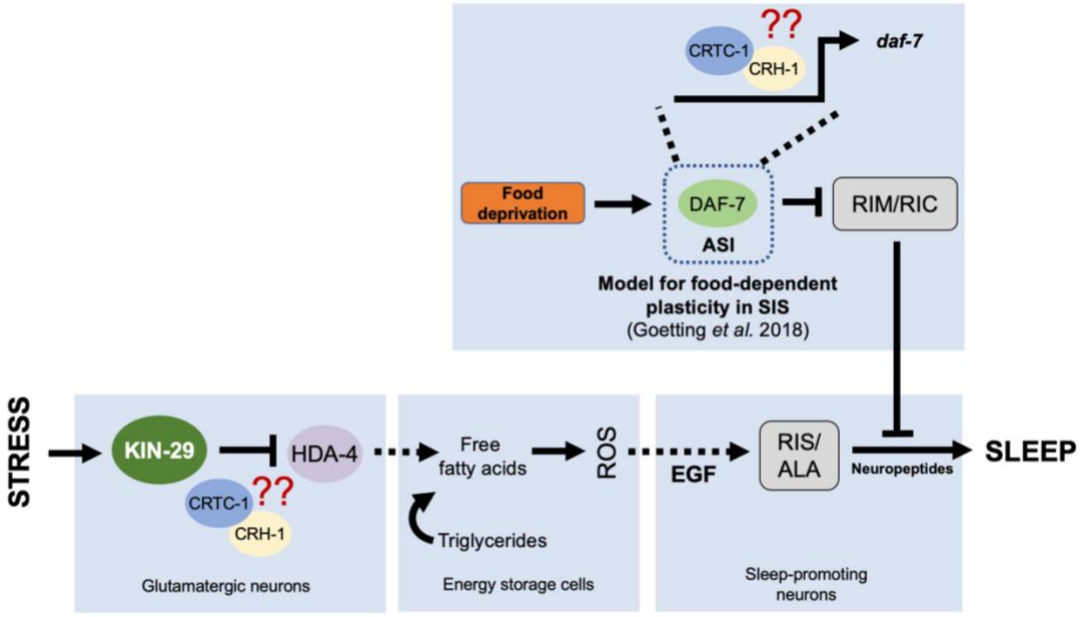


Figure 19. Potential model of CRTC1 in the regulation of SIS.

In response to stress, KIN-29/SIK phosphorylates HDA-4 and may act with CRTC-1 and CRH-1 in glutamatergic neurons to mobilize fat stores upstream of EGF and sleep promoting neurons RIS and ALA to regulate sleep. CRTC-1/CRH-1 may also act in the ASI neurons with DAF-7/TGF-β in the food-dependent plasticity pathway of SIS to regulate sleep.