The SEK-1 p38 MAP Kinase Pathway Regulates Gq Signaling in *C. elegans*

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Abstract

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Gq is a heterotrimeric G protein that is widely expressed in neurons and regulates neuronal activity. To identify pathways regulating neuronal Gq signaling we performed a forward genetic screen in *Caenorhabditis elegans* for suppressors of activated Gq. One of the suppressors is an allele of *sek-1*, which encodes a mitogen-activated protein kinase kinase (MAPKK) in the p38 MAPK pathway. We report that *sek-1* mutants have a slow locomotion rate. We show that *sek-1* acts in cholinergic neurons to regulate both locomotion and Gq signaling. Furthermore, we show that *sek-1* acts in mature neurons to regulate locomotion. We used genetic and behavioral approaches to demonstrate that other components of the p38 MAPK pathway also play a positive role in regulating locomotion and Gq signaling. There are two pathways downstream of Gq in *C. elegans* (PLCβ/EGL-8 and UNC-73/Trio) and we show that *sek-1* acts in the UNC-73/Trio pathway. Finally, we find that mutants in the *sek-1* p38 MAPK pathway partially

suppress an activated mutant of the sodium leak channel NCA-1/NALCN, a downstream target of Gq signaling. Our results suggest that the *sek-1* p38 pathway may modulate the output of Gq signaling through NCA-1.

Table of Contents

List of Figures and Tables

Glossary

AWC: Amphid wing “C” cells

C. elegans: *Caenorhabditis elegans*

EGL: Egg-laying defective

gf: gain-of-function

GEF: guanine exchange factor

GFP: green fluorescent protein

GPCR: G protein-coupled receptor

MAPK: mitogen-activated protein kinase

MAPKK: mitogen-activated protein kinase kinase

MAPKKK: mitogen-activated protein kinase kinase kinase

PLCβ: Phospholipase C β

SEK: SAPK(Stress-activated protein kinase)/ERK kinase

UNC: Uncoordinated

WGS: Whole genome sequencing

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Thank to you to my family and friends for supporting my grad school experience by discussing science, sending me rotten fruit (with wild *Caenorhabditis* inside), and sharing my appreciation of *C. elegans.*

Dedication

To Jordan Hoyt.

Thank you for being my collaborator for both Galaxy and life.

Chapter 1

Introduction

Neuronal Signaling and Gq

Neurons are the expert communicators of cells. Neurons receive many signals, integrate and transduce those signals, and ultimately send a signal to other cells. Molecules within cells are responsible for passing along signals. Heterotrimeric G proteins are a type of molecule within cells that are responsible for many types of signaling. Heterotrimeric G proteins receive signals from G protein-coupled receptors (GPCRs). GPCRs are activated in response to extracellular signals to the cell in the form of ligands, such as small molecule neurotransmitters and peptides. When G proteins are activated by GPCRs, the G proteins interact with downstream molecules to ultimately cause a cellular response to the signal, which in neurons is usually a signal sent to other cells.

Heterotrimeric G proteins consist of three components: Gα, Gβ, and Gγ. Humans have 16 Gα subunits, 3 Gβ subunits, and 14 Gγ subunits (Milligan and Kostenis, 2006).The heterotrimeric G protein complex exists at the plasma membrane, poised to respond to GPCR activation. This protein complex functions as a molecular switch. When the complex is inactive, all three of its components exist as a complex and the Gα is bound to GDP (Figure 1.1). When the GPCR

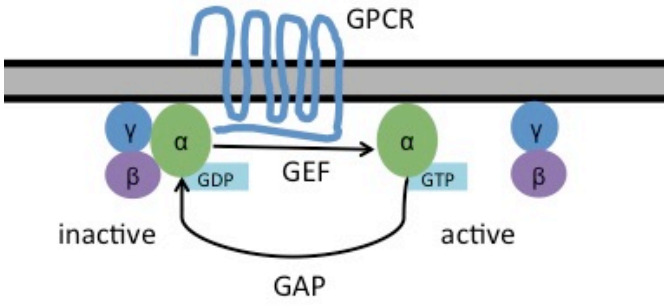

Figure 1.1: Summary of heterotrimeric G protein activation.

activates the complex, the GPCR acts as a guanine exchange factor (GEF) as it causes Gα to release GDP and instead bind GTP. The complex dissociates into Gα and a Gβγ dimer. Gα and Gβγ each have downstream effector molecules that they activate. There are four classes of Gα each with a set of downstream molecules that it can pass signals through: Gs, Gi/o, G12, and Gq. The human Gq class consists of Gq, G11, G14, and G15, and Gq is the most studied of these class members (Hubbard and Hepler, 2006).

Gq is expressed in many cell types and regulates many biological processes ranging from neurotransmission to cardiovascular physiology (Sánchez-Fernández et al., 2014). There are several pathways downstream of Gq. In the most studied pathway, activated Gq activates phospholipase Cβ (PLCβ) to cleave phosphatidylinositol 4,5-bisphosphate into diacylglycerol (DAG) and inositol triphosphate (IP_3) (Rhee, 2001). Gq also signals to kinases such as protein kinase Cζ and Bruton's tyrosine kinase (Btk) (Bence et al., 1997; García-Hoz et al., 2010). Kinases are a very important class of signaling molecules and these two kinases connect Gq activation to mitogen-activated protein kinase (MAPK) signaling cascades. Gq signaling also affects the monomeric G protein RhoA by interacting with its GEFs such as Trio (Vaqué et al., 2013). The diversity of Gq effectors showcases the plethora of pathways downstream of Gq and how one molecule has various effects in different cell types.

Disrupting signaling molecules alters the signaling pathway the molecule functions in and this alteration can cause diseases. There are many ways to disrupt signaling molecules, such as changing the presence of the molecules and also mutations within molecules that change the functional abilities. An activating mutation in Gq is reported as the cause of Sturge-Weber Syndrome, a skin and neurological

disorder, and this activated Gq causes increased signaling through the ERK/MAPK pathway (Shirley et al., 2013). A different activating mutation in Gq is found in some uveal melanomas and this mutated Gq also causes increased signaling in the ERK/MAPK pathway (Van Raamsdonk et al., 2009). Disrupting Gq signaling pathways by mutations in downstream signaling components also causes diseases and disorders. For example, two cases of early-onset epileptic encephalopathy are reportedly associated with loss of functional PLCβ (Kurian et al., 2010; Poduri et al., 2012). Studies in mice and other animals have purposefully disrupted Gq signaling to study the effects of downstream pathways. Mice have the same four Gq class members as humans (Wettschureck et al., 2004). Mice without Gq have impaired movement and mice lacking both Gq and G11 are not viable (Offermanns et al., 1997, 1998). To more easily study the pathway components involved in different Gq pathways, a system where there is only one Gq class member, but still conservation of signaling molecules, would be ideal.

Gq in *C. elegans*

The nematode *C. elegans* has a single Gq class member, EGL-30, and also conservation of Gq pathway components (Koelle, 2016). *C. elegans* is a great model organism to work with and is often referred to as “worms” within the scientific community. These worms are small (adults are 1 mm long), have a short lifecycle, and mutations within the genome create phenotypes and behaviors that can be observed by eye (Brenner, 1974). Additionally, the entire *C. elegans* genome has been sequenced (*C. elegans* Sequencing Consortium, 1998) and many techniques, such as CRISPR/Cas9 genome editing, have been adapted for use in *C. elegans* (Dickinson et al., 2013).

The *egl-30* gene that encodes EGL-30 was identified in *C. elegans* by looking for a gene that encoded certain conserved sequences present in Gq family members in other organisms (Brundage et al., 1996). Worms containing mutations in *egl-30* had been previously isolated and studied as they are defective in egg laying (Park and Horvitz, 1986; Trent et al., 1983). Brundage et al. (1996) identified the Gq gene in the same region and sequenced the Gq gene in some of the *egl-30* strains from the previous studies as well as some *egl-30* strains they generated. Their sequencing revealed mutations in the Gq gene in the *egl-30* strains. The authors then showed that the Gq gene is *egl-30* by expressing wild-type *egl-30* in *egl-30(ad809)* animals which rescued the *egl-30(ad809)* egg-laying-deficient phenotype.

Worms with mutations in *egl-30* have other phenotypes aside from deficient egg laying. Brundage et al. (1996) reported phenotypes and changes in the EGL-30 protein of many *egl-30* alleles such as *ad810* and *ad805*, which are both loss-of-function alleles. *ad810* causes larval arrest in worms, and is thought to be a null allele. Worms with *egl-30(ad805)* are viable but sluggish and and leave shallow tracks in the bacterial lawn that worms live on. Interestingly, overexpression of wild-type *egl-30* also creates visible phenotypes of laying eggs early and leaving deep tracks (Brundage et al., 1996). *egl-30* gain-of-function alleles also cause deep body bends and hyperactive locomotion (Bastiani et al., 2003; Doi and Iwasaki, 2002; Hawasli et al., 2004). The *tg26* allele (R243Q) likely alters guanine nucleotide binding (Bastiani et al., 2003; Doi and Iwasaki, 2002). The *js126* allele (V180M) exists in a region which is implicated in GTP hydrolysis (Hawasli et al., 2004). These studies show that *egl-30* is required for wild-type locomotion and aberrant signaling by mutated EGL-30 causes locomotion phenotypes.

In *C. elegans*, EGL-30 has multiple downstream signaling pathways. EGL-30 signals through PLCβ (EGL-8), as Gq does in other systems (Lackner et al., 1999). EGL-8 is not the sole EGL-30 effector as null mutations in *egl-8* do not cause the same severe phenotype as the null *egl-30(ad810)* (Lackner et al., 1999). EGL-30 also signals through the RhoGEF Trio (UNC-73) and worms containing null mutations in both *egl-8* and *unc-73* phenocopy the null *egl-30* worms. UNC-73 activates RHO-1 (the ortholog of RhoA), which has been shown to enhance neurotransmitter release through both diacylglycerol kinase (DGK-1)-dependent and DGK-1-independent pathways (McMullan et al., 2006).

Relatively little is known about neuronal EGL-30/Gq signaling that is downstream of an effector other than EGL-8/PLCβ. To identify additional signaling molecules, we used the *egl-30(tg26)* gain-of-function (hereafter referred to as *egl-30(gf)*) worms and performed a forward genetic screen. Since *egl-30(gf)* causes increased signaling from EGL-30 resulting in hyperactive locomotion and other phenotypes, mutations in downstream signaling molecules should result in less signaling through pathways downstream of Gq and suppress *egl-30(gf)* phenotypes. From our screen, we isolated over a hundred suppressors of *egl-30(gf)*. Some of these mutations were in *egl-30*, *egl-8*, and *unc-73*, affirming that we screened for mutations that would reduce EGL-30 signaling.

One of the mutants isolated from the *egl-30(gf)* suppressor screen was *yak42*. We performed whole genome sequencing and found that *yak42* is a mutation in *sek-1*. *sek-1* encodes SEK-1, a MAPK kinase known to be involved in signal transduction (Kim et al., 2002; Tanaka-Hino et al., 2002).

MAPK signaling pathways

Mitogen-activated protein kinases (MAPK) are involved in many different signal transduction pathways. There are three major classes of MAPKs: ERK (extracellular signal-related kinase), JNK (c-Jun N-terminal protein kinase), and p38 (as it is a 38 kDa protein). Each MAPK has a variety of target proteins but one common MAPK feature is the kinase cascade leading to its activation. MAPKs are phosphorylated by MAPKKs (MAPK Kinases), which are phosphorylated by MAPKKKs (MAPKK Kinases) (Figure 1.2). These MAPK signaling pathways are activated in response to various stresses and chemical signals and affect many processes such as gene expression, cell proliferation, and inflammation. With all these different signals and outputs, one could imagine a chaotic cellular signaling landscape. However, no signaling happens in a vacuum so context (such as the cell type or location of signaling within the cell) ensures that a certain signal is transduced through a MAPK pathway to result in specific responses rather than affecting all possible downstream processes.

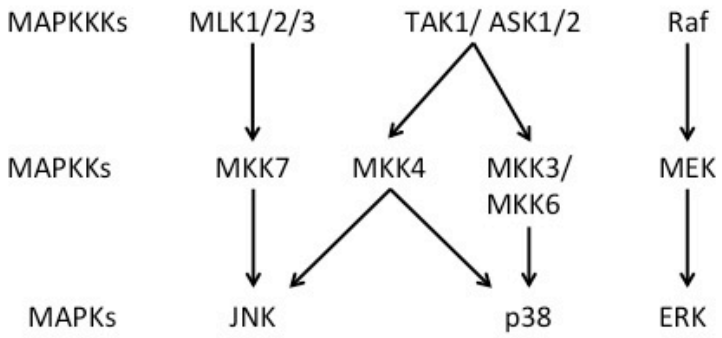

Figure 1.2: MAPK signaling cascades. MAPKKKs phosphorylate MAPKKs, which phosphorylate MAPKs. Each MAPK pathway is named after the MAPK involved.

Of the three MAPK pathways, the ERK/MAPK pathway is the most studied. The ERK MAPK pathway promoted cellular proliferation and mutations in the ERK MAPK pathway are found in many types of cancer. ERK MAPK is activated by MEK, a MAPKK that is activated by a Raf MAPKKK (Dhillon et al., 2007) (Figure 1.2).The other two MAPK pathways are JNK and p38, which are known as stress-activated protein kinases. JNK is activated by both MKK7 and MKK4 (Wang et al., 2007). MKK4 can also contribute to the activation of p38 in certain situations (Brancho et al., 2003).

The p38 MAPK pathway is activated by a variety of cellular stresses and inflammatory cytokines (Cuenda and Rousseau, 2007). p38 is activated by MKK3, MKK6, and MKK4 (Zarubin and Han, 2005). The *C. elegans* SEK-1 is an ortholog of MKK3/6 (Tanaka-Hino et al., 2002). Mice lacking either MKK3 or MKK6 are healthy (Lu et al., 1999; Tanaka et al., 2002) but mice lacking both are not viable and die before birth (Brancho et al., 2003).

There have been a few reports connecting Gq signaling and the p38 MAPK pathway. p38 has also been shown to be activated downstream of a GPCR in rat neurons (Huang et al., 2004). While Gq has been shown to signal to the p38 MAPK

pathway through Btk (Bence et al., 1997), *C. elegans* do not have an ortholog of Btk or any member of its Tec family of kinases (Plowman et al., 1999).

SEK-1 in *C. elegans*

sek-1 was identified in *C. elegans* by the Matsumoto group (Tanaka-Hino et al., 2002). They first identified a JNK MAPK that they named JNK-1 and the kinase that activates JNK-1, a MAPKK named JKK-1 (Kawasaki et al., 1999). This group also identified SEK-1 as JNK-1 activator. They showed this by expressing both JNK-1 and SEK-1 in yeast lacking both the yeast orthologs JNK-1 and JNK-1 Kinase, so this is an interaction that may not happen in worms. The Matsumoto group collaborated with the Bargmann group to characterize the role of SEK-1 in the development of asymmetric neurons (Tanaka-Hino et al., 2002). SEK-1 has high sequence similarity to two classes of MAPKKs: 50% identical to MKK3/6 and 43% identical to MKK4. SEK-1 was also shown to activate p38. MKK3 and MKK6 act as the MAPKK for p38 but MKK4 can phosphorylate both p38 and JNK MAPK (Figure 1.2). There are no reports of SEK-1 activating JNK-1 in *C. elegans* and SEK-1 is regarded as MKK3/6 ortholog.

The SEK-1 protein is encoded by the gene *sek-1,* which is located on chromosome X and made up of seven exons. Tanaka-Hino et al. (2002) created the *km4* allele of *sek-1*, which deletes exons 3-6 which encode many kinase subdomains so this deletion allele is presumed to be a null allele. They used this allele to study the development of the asymmetric Amphid wing “C” (AWC) olfactory neurons. In wild-type *C. elegans*, only one of the two AWC neurons expresses STR-2 (an olfactory receptor) (Troemel et al., 1999), while *sek-1(km4)* worms express STR-2 in both AWC neurons.

Since SEK-1 is a MAPKK and therefore activated by phosphorylation, the authors made a mutant *sek-1* that would mimic phosphorylation and it resulted in no STR-2 expression in the AWC neurons. SEK-1 was shown to function downstream of NSY-1, a MAPKKK that is the *C. elegans* ASK1 homolog, but no MAPK was identified. The authors reported that *sek-1* is expressed in many tissues and neurons. Furthermore, they showed that SEK-1 acts in the AWC neurons to regulate asymmetric development by rescuing the *sek-1(km4)* AWC phenotype with wild-type *sek-1* expressed by an olfactory neuron promoter. This study identified *sek-1* and showed that that SEK-1 is a MAPKK that acts in neurons downstream of NSY-1 (Figure 1.3; Tanaka-Hino et al., 2002).

SEK-1 was also discovered for its role in innate immunity (Kim et al., 2002). Kim et al. (2002) screened for *C. elegans* with enhanced susceptibility to pathogens (Esp) and identified several mutants with this Esp phenotype, including *esp-2(ag1).* Expression of wild-type *sek-1* rescued the Esp phenotype of *esp-2(ag1)*, showing that *esp-2* is *sek-1*. *ag1* is a single nucleotide polymorphism that causes G212R. Animals with *sek-1(km4)* also exhibited a Esp phenotype. Additionally, *esp-8(ag3)* was shown to create a stop codon in NSY-1, the MAPKKK that acts with SEK-1 in the specification of AWC neurons. This study also identified PMK-1 as the MAPK by knocking down *pmk-1* expression with RNAi rather then using a mutant *pmk-1* as no mutants were available at the time (Kim et al., 2002).

There are many parallels between these early studies of *sek-1* as they all identify NSY-1 as the MAPKKK involved in these processes. Studies focused on these different fields of immunity and AWC neuronal specification led to more findings that fill out the NSY-1-SEK-1 signaling module. It was recently found that SEK-1 targets both PMK-1

and PMK-2 in AWC neuronal specification as these two p38 orthologs function redundantly in the *C. elegans* nervous system (Figure 1.3; Pagano et al., 2015). For both innate immunity and AWC specification, the p38 MAPK pathway acts downstream of the adaptor protein TIR-1 (an ortholog of SARM) (Chuang and Bargmann, 2005; Couillault et al., 2004). These studies established a *C. elegans* p38 MAPK signaling module that consists of TIR-1, NSY-1, and SEK-1.

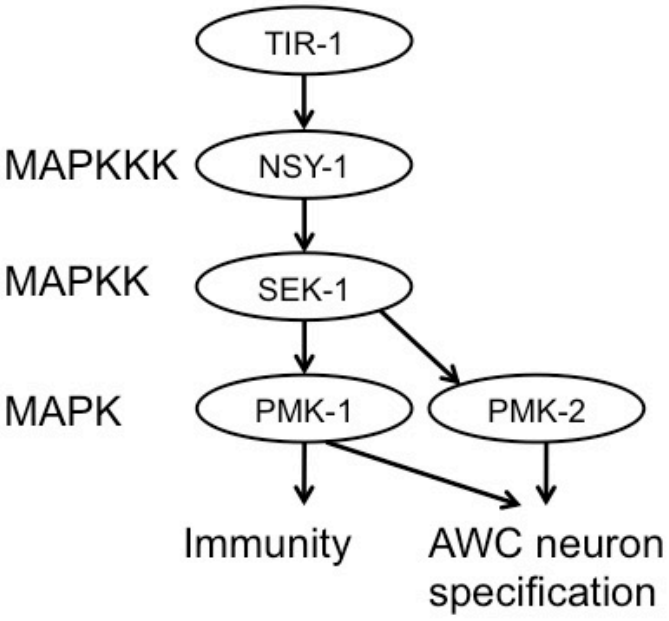

Figure 1.3: TIR-1 and the p38 MAPK signaling pathway in *C. elegans*. TIR-1 acts upstream of the p38 MAPK pathway which consists of NSY-1 (MAPKKK), SEK-1 (MAPKK), and PMK-1, PMK-2 (p38 MAPKs). Two known roles of this signaling module are in immunity and AWC neuron specification.

sek-1(yak42) was isolated in a screen for suppressors of activated Gq in *C. elegans*. I employed several genetic, behavioral analysis, and molecular biology techniques to show that SEK-1 acts in mature cholinergic neurons to regulate locomotion and also that the entire TIR-1/NSY-1/SEK-1/PMK-1 PMK-2 signaling module acts downstream of Gq.

Chapter 2

The SEK-1 p38 MAPK pathway regulates Gq signaling in *C. elegans*

Introduction

Gq is a widely expressed heterotrimeric G protein that regulates a variety of biological processes ranging from neurotransmission to cardiovascular physiology (Sánchez-Fernández et al., 2014). In the canonical Gq pathway, Gq activates phospholipase Cβ (PLCβ), which cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into the second messengers diacylglycerol (DAG) and inositol trisphosphate (IP_3) (Rhee, 2001). In addition to PLCβ, other Gq effectors have been identified including kinases, such as protein kinase Cζ (PKCζ) and Bruton's tyrosine kinase (Btk) (Bence et al., 1997; García-Hoz et al., 2010), and guanine nucleotide exchange factors (GEFs) for the small GTPase Rho, such as Trio (Williams et al., 2007; Vaqué et al., 2013). These noncanonical effectors bridge the activation of Gq to other cellular signaling cascades.

To study noncanonical pathways downstream of Gq, we used the nematode *C. elegans* which has a single Gαq homolog (EGL-30) and conservation of the other components of the Gq signaling pathway (Koelle, 2016). In neurons, EGL-30 signals through EGL-8 (PLCβ) (Lackner et al., 1999) and UNC-73 (ortholog of Trio RhoGEF) (Williams et al., 2007). UNC-73 activates RHO-1 (ortholog of RhoA), which has been shown to enhance neurotransmitter release through both diacylglycerol kinase (DGK-1)-dependent and DGK-1-independent pathways (McMullan et al., 2006).

To identify additional signaling pathways that modulate Gq signaling, we screened for suppressors of the activated Gq mutant *egl-30(tg26)* (Doi and Iwasaki, 2002),

hereafter referred to as *egl-30(gf). egl-30(gf)* mutant animals exhibit hyperactive locomotion and a “loopy” posture in which worms have exaggerated, deep body bends and loop onto themselves (Bastiani et al., 2003). Here we identify one of the suppressors as a deletion allele in the gene *sek-1*. SEK-1 is a mitogen-activated protein kinase kinase (MAPKK), the *C. elegans* ortholog of mammalian MKK3/6 in the p38 MAPK pathway (Tanaka-Hino et al., 2002). The p38 MAPK pathway has been best characterized as a pathway activated by a variety of cellular stresses and inflammatory cytokines (Kyriakis and Avruch, 2012). However, the p38 MAPK pathway has also been shown to be activated downstream of a G protein-coupled receptor in rat neurons (Huang et al., 2004). Btk, a member of the Tec family of tyrosine kinases, has been shown to act downstream of Gq to activate the p38 MAPK pathway (Bence et al., 1997), but *C. elegans* lacks Btk and other Tec family members (Plowman et al., 1999).

SEK-1 is activated by the MAPKKK NSY-1 (ortholog of ASK1) and activates the p38 MAPKs PMK-1 and PMK-2 (Andrusiak and Jin, 2016). The p38 MAPK pathway consisting of NSY-1/SEK-1/PMK-1 is required for innate immunity in *C. elegans* (Kim et al., 2002). NSY-1 and SEK-1 are also required for the specification of the asymmetric AWC olfactory neurons (Sagasti et al., 2001; Tanaka-Hino et al., 2002); the p38 orthologs PMK-1 and PMK-2 function redundantly in AWC specification (Pagano et al., 2015). For both innate immunity and AWC specification, the p38 MAPK pathway acts downstream of the adaptor protein TIR-1 (an ortholog of SARM) (Couillault et al., 2004; Chuang and Bargmann, 2005). Here we show that the TIR-1/NSY-1/SEK-1/PMK-1 PMK-2 signaling module also acts to regulate locomotion downstream of Gq signaling.

Results

sek-1 suppresses activated Gq

To identify downstream effectors of Gαq, we performed a forward genetic screen for suppressors of the activated Gq mutant, *egl-30(tg26)* (Doi and Iwasaki, 2002), referred to here as *egl-30(gf). egl-30(gf)* worms are small, hyperactive, and have a "loopy" posture characterized by a high-amplitude waveform (Figure 2.1B). Thus, we screened for worms that are larger, less hyperactive, and less loopy. We isolated a recessive suppressor, *yak42*, and mapped it to the middle of Chromosome X (see Materials and Methods). Whole-genome sequencing revealed that *yak42* carries a large deletion of the *sek-1* gene from upstream of the start codon to exon 4 (Figure 2.1A). *yak42* also failed to complement *sek-1(km4)*, a previously published *sek-1* deletion allele (Figure 2.1A) (Tanaka-Hino et al., 2002).

egl-30(gf) double mutants with either *sek-1(yak42)* or *sek-1(km4)* are bigger than *egl-30(gf)* worms (Figure 2.1B), not hyperactive (Figure 2.1C) and have a tendency to kink when moving backward. Additionally, both *sek-1* mutations suppress the loopy waveform phenotype (Figure 2.1D, E). To test whether the *egl-30(gf)* suppression phenotype might be an indirect effect of the slow locomotion of a *sek-1* mutant, we built an *egl-30(gf)* double mutant with a mutation in *unc-82*, a gene required for normal muscle structure. *unc-82* mutants are coordinated but move slowly, similar to a *sek-1* mutant (Hoppe et al., 2010). However, although an *egl-30(gf) unc-82(e1220)* double mutant moves more slowly than *egl-30(gf)*, it is still small and loopy (Figure 2.1B).

EGL-30/Gαq is negatively regulated by GOA-1, the worm Gαo/i ortholog (Hajdu-Cronin et al., 1999). We tested whether *sek-1* also suppresses a *goa-1* loss-of-function

mutant that causes a hyperactive phenotype similar to *egl-30(gf).* Indeed, *sek-1(km4)* suppresses *goa-1(sa734)* (Figure 2.1F). One downstream effector of GOA-1 is the DAG kinase DGK-1 (DGKΘ ortholog) that inhibits DAG-dependent functions such as synaptic vesicle release (Miller et al., 1999; Nurrish et al., 1999). *dgk-1(sy428)* animals are hyperactive, but *sek-1(km4)* does not suppress *dgk-1*. Rather, the *sek-1 dgk-1* double mutant is uncoordinated and looks like neither *sek-1* nor *dgk-1* mutants, confounding the interpretation of how *sek-1* genetically interacts with *dgk-1*.

sek-1(yak42) was outcrossed from *egl-30(gf)* and assayed for locomotion defects. Both the *sek-1(yak42)* and *sek-1(km4)* mutants are coordinated but move more slowly than wild-type (Figure 2.1G). *sek-1(ag1)*, a point mutation in exon 5 (Kim et al., 2002), also causes a similar slow locomotion phenotype (data not shown).

Thus, *sek-1* appears to be a specific suppressor of multiple phenotypes of the *egl-30(gf)* mutant, suggesting that *sek-1* regulates locomotion and acts downstream of Gq.

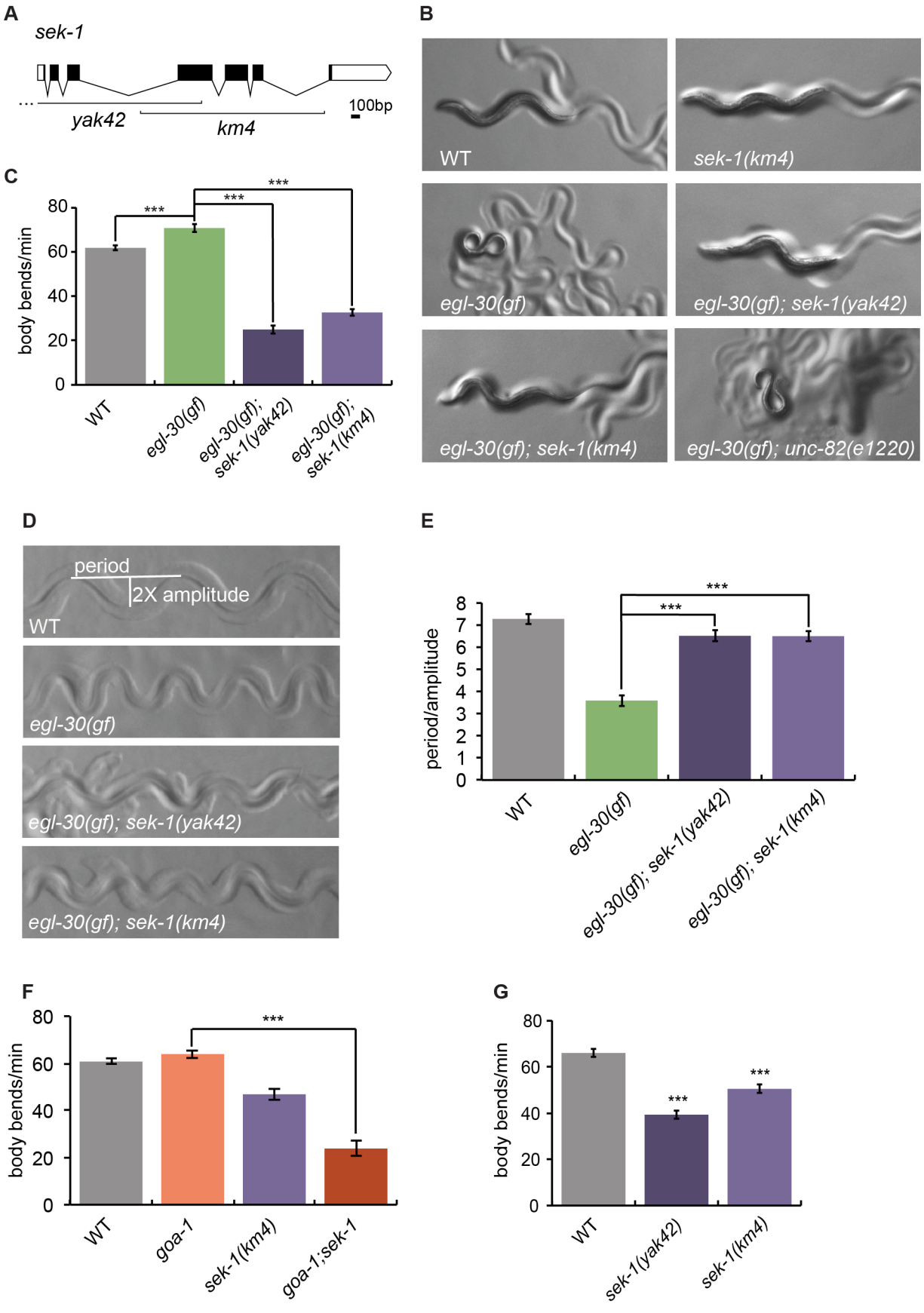
A
sek-1
yak42
km4
100bp
B
WT
sek-1(km4)
egl-30(gf)
egl-30(gf); sek-1(yak42)
egl-30(gf); sek-1(km4)
egl-30(gf); unc-82(e1220)
C
body bends/min
0
20
40
60
80
***
***
***
WT
egl-30(gf)
egl-30(gf); sek-1(yak42)
egl-30(gf); sek-1(km4)
D
period
2X amplitude
WT
egl-30(gf)
egl-30(gf); sek-1(yak42)
egl-30(gf); sek-1(km4)
E
period/amplitude
0
1
2
3
4
5
6
7
8
***
***
WT
egl-30(gf)
egl-30(gf); sek-1(yak42)
egl-30(gf); sek-1(km4)
F
body bends/min
0
20
40
60
80
***
WT
goa-1
sek-1(km4)
goa-1;sek-1
G
body bends/min
0
20
40
60
80
***
***
WT
sek-1(yak42)
sek-1(km4)

Figure 2.1: *sek-1* acts downstream of Gαq to regulate locomotion behavior
(A) Gene structure of *sek-1*. White boxes depict the 5' and 3' untranslated regions, black boxes depict exons, and lines show introns. The positions of the *yak42* and *km4* deletions are shown. *yak42* is a 3713 bp deletion that extends to 1926 bp upstream of the start codon. Drawn with Exon-Intron Graphic Maker (http://www.wormweb.org/exonintron). Scale bar is 100 bp.
(B) *sek-1(yak42)* and *sek-1(km4)* suppress the small size and loopy waveform of *egl-30(tg26)*, written here as *egl-30(gf). unc-82(e1220)* does not suppress *egl-30(gf).* Photos of first-day adult worms. WT: wild type.
(C) *egl-30(gf)* is hyperactive compared to wild-type and suppressed by *sek-1(yak42)* and *sek-1(km4)*. ***, p<0.001, error bars = SEM, n=20.
(D) *egl-30(gf)* mutants have deeper body bends than wild-type (WT) and *sek-1* mutations suppresses this loopy posture. Images show tracks of forward-moving first-day adults.
(E) Quantification of the waveform phenotype. ***, p<0.001, error bars = SEM, n=4-5.
(F) *sek-1* suppresses *goa-1* hyperactivity. *goa-1(sa734) sek-1(km4)* animals move more slowly than *goa-1(sa734)* animals. ***, p<0.001, error bars = SEM, n=20.
(G) *sek-1* mutant worms have slow locomotion. ***, p<0.001 compared to wild-type, error bars = SEM, n=20.

sek-1 acts in mature cholinergic neurons to regulate locomotion

egl-30 is widely expressed and acts in neurons to regulate locomotion (Lackner et al., 1999), so it is possible that *sek-1* also acts in neurons to regulate Gq signaling. *sek-1* is expressed in neurons, intestine, and several other tissues (Tanaka-Hino et al., 2002) and has been shown to function in GABA neurons to possibly promote GABA release (Vashlishan et al., 2008). To identify the cell type responsible for the *sek-1* locomotion phenotypes, we expressed the wild-type *sek-1* cDNA under different cell-specific promoters and tested for transgenic rescue of a *sek-1* null mutant. Expression of *sek-1* in all neurons (using the *unc-119* promoter) or in cholinergic neurons (using the *unc-17* promoter) was sufficient to rescue the *sek-1* mutant slow locomotion phenotype, but expression in GABA neurons (using the *unc-47* promoter) was not sufficient to rescue (Figure 2.2 A, B). These results indicate that *sek-1* acts in cholinergic neurons to regulate locomotion.

Because *sek-1* acts in the development of the AWC asymmetric neurons, we asked whether *sek-1* also has a developmental role in regulating locomotion. We induced *sek-1* expression (using a heatshock-inducible promoter) to test if *sek-1* expression in mature neurons is sufficient to rescue the *sek-1* mutant. We found that *sek-1* expression in adults rescues the *sek-1* slow locomotion phenotype (Figure 2.2 C). This result indicates that *sek-1* is not required for development of the locomotion circuit and instead acts in mature neurons to regulate locomotion.

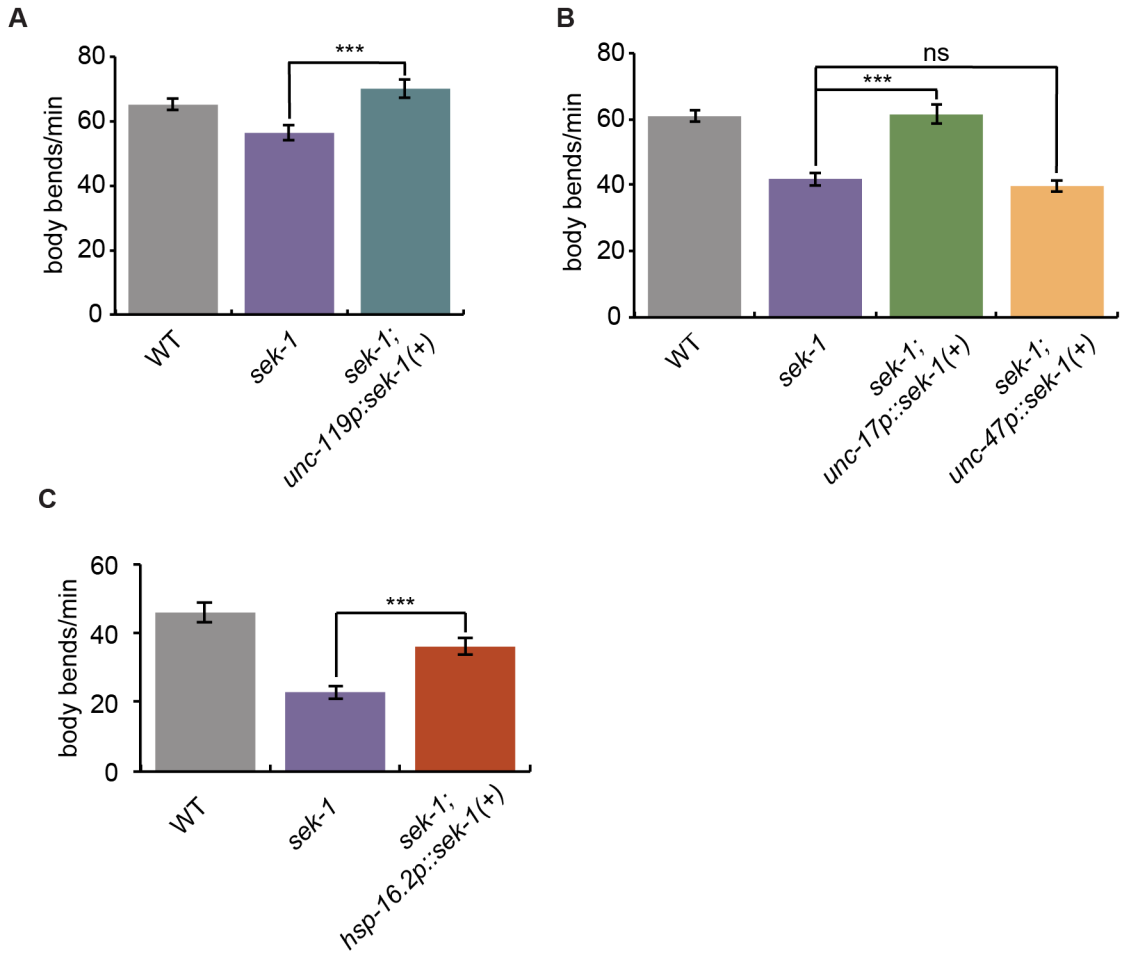

Figure 2.2: *sek-1* acts in mature cholinergic neurons to regulate locomotion
(A) *sek-1* acts in neurons to regulate locomotion. The *sek-1* wild-type cDNA driven by the *unc-119* pan-neuronal promoter *[unc-119p::sek-1(+)]* rescues the slow locomotion phenotype of *sek-1(km4)* worms. ***, p< 0.001, error bars = SEM, n=20.

(B) *sek-1* acts in cholinergic neurons to regulate locomotion. *sek-1* WT cDNA driven by the *unc-17* cholinergic neuron promoter *[unc-17p::sek-1(+)]* rescues the slow locomotion phenotype of *sek-1(km4)* worms but *sek-1* expression in GABA neurons using the *unc-47* promoter *[unc-47p::sek-1(+)]* does not. p< 0.001, error bars = SEM, n=20.
(C) *sek-1* acts in mature neurons to regulate locomotion. *hsp-16.2p::sek-1(+)* rescues the slow locomotion phenotype of *sek-1(km4)*. ***, p< 0.001, error bars = SEM, n=20.

sek-1* acts in cholinergic neurons to suppress *egl-30(gf)

We next tested whether *sek-1* also acts in neurons to suppress *egl-30(gf)*. Expression of *sek-1* under pan-neuronal and cholinergic neuron promoters rescued the *sek-1* suppression of *egl-30(gf)*. Specifically, *egl-30(gf) sek-1* double mutants expressing wild-type *sek-1* in all neurons or cholinergic neurons had a hyperactive, loopy, small phenotype that resembled the *egl-30(gf)* single mutant (Figure 2.3 A, B). However, expression of *sek-1* in GABA neurons did not rescue the suppression phenotype (Figure 2.3 A, B). Together, these data show that *sek-1* acts in cholinergic neurons to regulate both wild-type locomotion and to modulate Gq signaling.

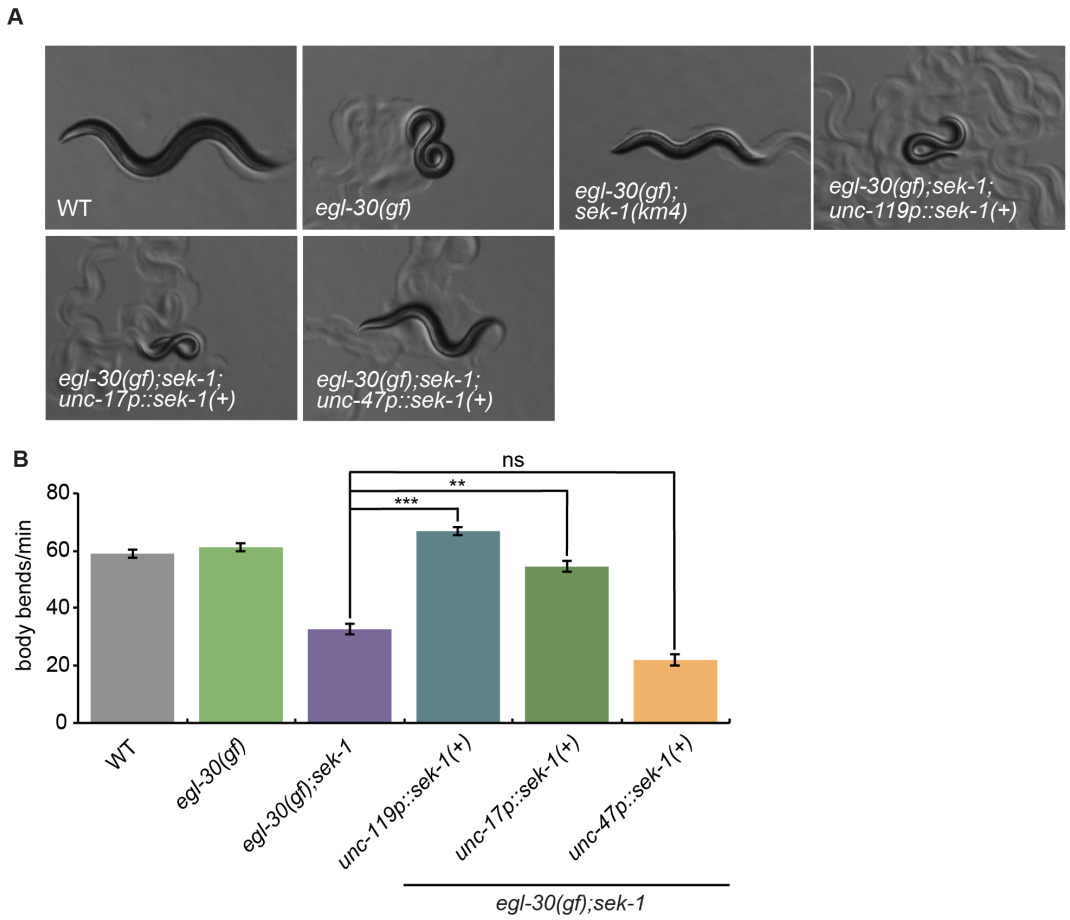

Figure 2.3: *sek-1* acts in cholinergic neurons to suppress *egl-30(gf)*
(A-B) Worms expressing *unc-119p::sek-1(+)* or *unc-17p::sek-1(+)* do not have the *egl-30(gf) sek-1* phenotype but worms expressing *unc-47p::sek-1(+)* are identical to *egl-30(gf) sek-1* both for waveform (A) and in the locomotion assay (B). Kruskal-Wallis test, ***, p< 0.001, **, p<0.01; error bars = SEM, n=17-20.

The p38 MAPK pathway is a positive regulator of Gq signaling

SEK-1 is the MAPKK in a p38 MAPK pathway (Tanaka-Hino et al., 2002). This pathway consists of NSY-1 (MAPKKK), SEK-1 (MAPKK), and PMK-1 or PMK-2 (MAPKs)(Andrusiak and Jin, 2016). TIR-1 acts upstream of NSY-1. This p38 MAPK signaling module has been shown to function in innate immunity and the development of the AWC olfactory neurons (Chuang and Bargmann, 2005).

We tested whether the entire p38 MAPK and TIR-1 signaling module also regulates locomotion and suppression of activated Gq. Both *tir-1(tm3036)* and *nsy-1(ok593)* mutant animals have slow locomotion on their own and also suppress the hyperactivity, deep body bends and small size of *egl-30(gf)* (Figure 2.4 A-D). We also tested single mutants in each of the three worm p38 MAPK genes (*pmk-1, pmk-2* and *pmk-3)* and a *pmk-2 pmk-1* double mutant. Although we found that the *pmk-2* and *pmk-3* single mutants were slightly slow on their own, only the *pmk-2 pmk-1* double mutant phenocopied *sek-1* and suppressed both the hyperactivity and deep body bends of *egl-30(gf)* (Figure 2.4, E-G). Thus, *pmk-2* and *pmk-1* act redundantly downstream of *sek-1* to suppress *egl-30(gf).* These data suggest that the p38 MAPK pathway regulates locomotion in *C. elegans* and acts genetically downstream of *egl-30*.

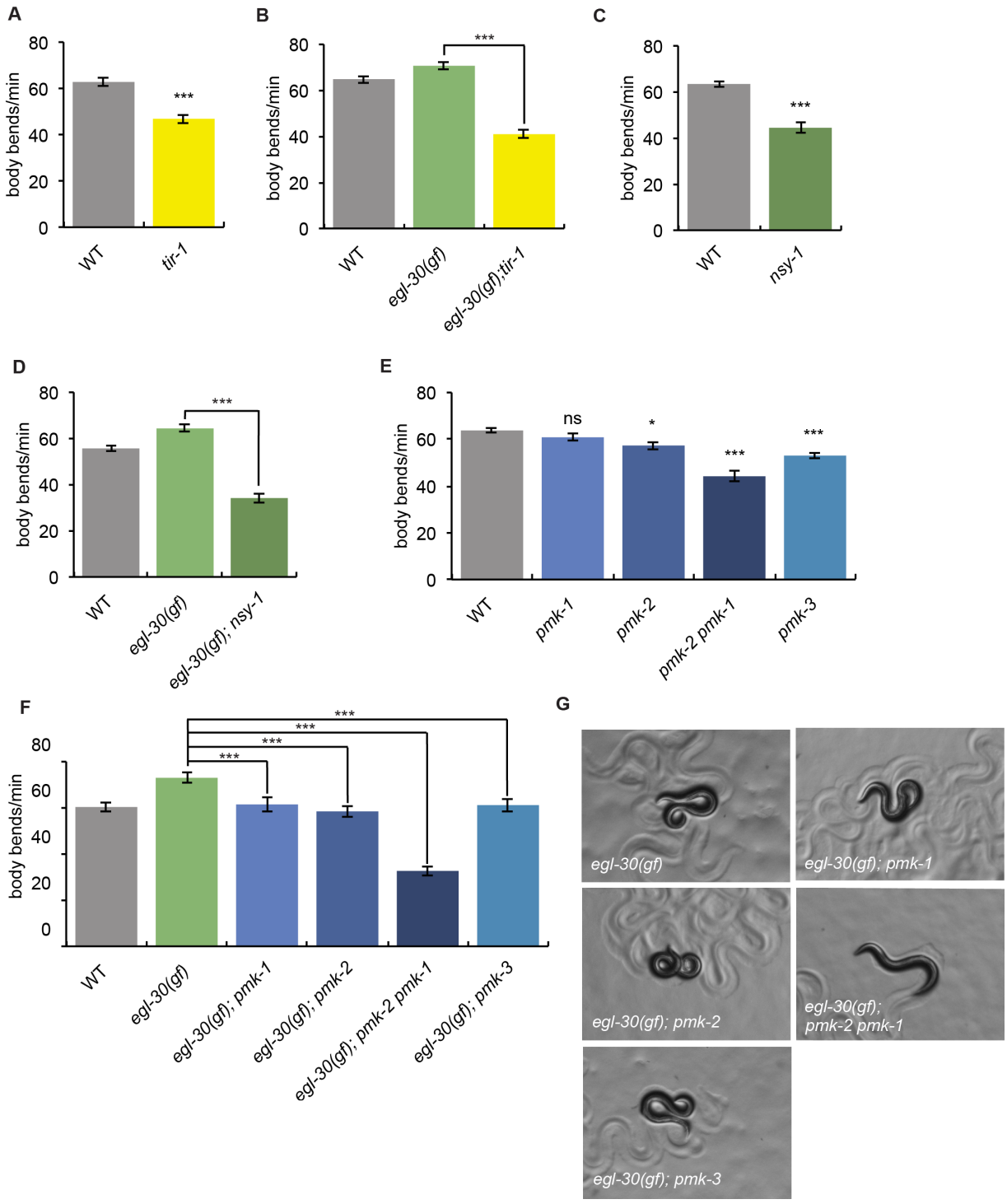

Figure 2.4: The p38 MAPK pathway regulates locomotion downstream of *egl-30*
(A) *tir-1(tm3036)* mutant animals have slow locomotion. ***, p<0.001, error bars = SEM, n=20.
(B) *tir-1(tm3036)* suppresses *egl-30(gf)*. *egl-30(gf) tir-1* animals move more slowly than the hyperactive *egl-30(gf)* animals. ***, p< 0.001, error bars = SEM, n=20.

(C) *nsy-1(ok593)* mutant animals have slow locomotion. ***, p<0.001, error bars = SEM, n=20.
(D) *nsy-1(ok593)* suppresses *egl-30(gf)*. *egl-30(gf) nsy-1* animals move more slowly than the hyperactive *egl-30(gf)* animals. ***, p< 0.001, error bars = SEM, n=20.
(E) *pmk-2, pmk-2 pmk-1,* and *pmk-3* mutant animals have slow locomotion. ***, p< 0.001; *, p<0.05, compared to WT, error bars = SEM, n=20.
(F) A *pmk-2 pmk-1* double mutant suppresses the hyperactivity of *egl-30(gf)*. ***, p< 0.001, error bars = SEM, n=20.
(G) A *pmk-2 pmk-1* double mutant suppresses the deep body bends of *egl-30(gf)*. *egl-30(gf)* animals with mutations in either *pmk-1, pmk-2,* or *pmk-3* are still small and loopy. *egl-30(gf) pmk-2 pmk-1* animals are less loopy and have a more wild-type posture.

Exploration of other possible regulators of Gq signaling

The JNK MAPK pathway, related to the p38 MAPK family, also regulates locomotion in *C. elegans*. Specifically, the JNK pathway members *jkk-1* (JNK MAPKK) and *jnk-1* (JNK MAPK) have been shown to act in GABA neurons to regulate locomotion (Kawasaki et al., 1999). We found that the *jkk-1* and *jnk-1* single mutants had slow locomotion and that the double mutants with p38 MAPK pathway members exhibited an additive slow locomotion phenotype (Figure 2.5 A). Moreover, neither *jkk-1* nor *jnk-1* suppressed *egl-30(gf)* (data not shown). Thus, the JNK and p38 MAPK pathways regulate locomotion independently and the JNK pathway is not involved in Gq signaling.

We also tested the involvement of possible p38 MAPK pathway effectors. One of the targets of PMK-1 is the transcription factor ATF-7 (Shivers et al., 2010). Both the *atf-7(qd22 qd130)* loss-of-function mutant and the *atf-7(qd22)* gain-of-function mutant moved slowly compared to wild-type animals (Figure 2.5 B). However, *atf-7(qd22 qd130)* did not suppress *egl-30(gf)* (data not shown), suggesting that *atf-7* is not a target of this pathway or acts redundantly with other downstream p38 MAPK targets. We also tested *gap-2*, the closest *C. elegans* homolog of ASK1-interacting Protein (AIP1) which

activates ASK1 (the ortholog of *C. elegans* NSY-1) in mammalian systems (Zhang et al., 2003). A *C. elegans gap-2* mutant has no locomotion defect (Figure 2.5 C). Finally, we tested VHP-1, a phosphatase for p38 and JNK MAPKs that inhibits p38 MAPK signaling (Kim et al., 2004). However, the *vhp-1(sa366)* mutant also has no locomotion defect (Figure 2.5 C).

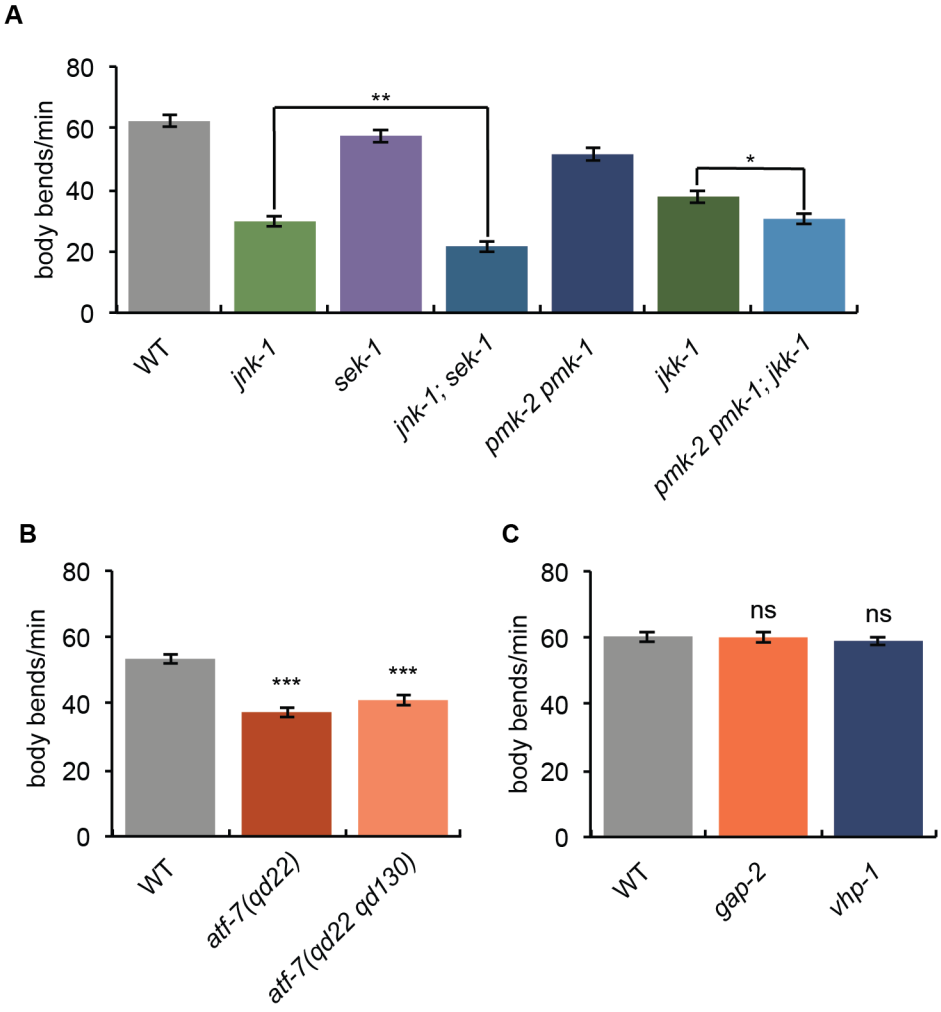

Figure 2.5: The JNK MAPK pathway and p38 MAPK pathway effectors do not regulate locomotion like the p38 MAPK pathway
(A) *jkk-1* and *jnk-1* act in parallel to *sek-1* and *pmk-2 pmk-1*. The *jnk-1(gk7) sek-1(km4)* double mutant and *pmk-2(qd279 qd171) pmk-1(km25) jkk-1(km2)* triple mutants move more slowly than the respective individual mutants. **, p< 0.01; *, p<0.05; error bars = SEM, n=20.

(B) Worms with gain-of-function or loss-of-function alleles of *atf-7* are slower than wild-type worms. $p < 0.001$, error bars = SEM, n=20.
(C) Worms lacking *gap-2* and *vhp-1* move like wild-type worms. Neither *gap-2(tm478)* nor *vhp-1(sa366)* confers a slow locomotion phenotype. ns, $p > 0.05$ compared to WT, error bars = SEM, n=20.

Activating the p38 pathway results in a slow locomotion phenotype

egl-30(gf) animals are loopy and hyperactive so we tested whether increased activation of the TIR-1/p38 MAPK signaling module causes similar phenotypes. The *tir-1(ky648gf)* allele leads to a gain-of-function phenotype in the AWC neuron specification (Chang et al., 2011). However, *tir-1(ky648gf)* does not cause a locomotion phenotype reminiscent of *egl-30(gf)*, as these worms look similar to wild-type worms and move slower than wild-type (Figure 2.6 A, B).

We also created a gain-of-function of *sek-1* using the CRISPR/Cas9 genome editing system (Dickinson et al., 2013). NSY-1 phosphorylates SEK-1 on S204 and T208, therefore making phosphomimetic mutations (SEK-1: S204D T208D) results in a gain-of-function phenotype (Tanaka et al., 2002). Tanaka et al. (2002) expressed SEK-1(S204D T208D) in olfactory neurons. We wanted a global gain-of-function *sek-1* allele so we edited the endogenous *sek-1* gene to express *sek*-1(S204D T208D) (officially *sek-1(yak115)*). S204 and T208 are encoded by adjacent exons, so we replaced the intron (originally 151 bp) between these exons with a selection marker (making the intron 2148 bp). *sek*-1(S204D T208D) worms exhibited a slow locomotion phenotype like *sek-1(km4)* (Figure 2.6 C). Unfortunately, these *sek-1*(S204D T208D) worms expressed STR-2 in both AWC neurons indicating that *yak115* is a loss-of-function allele, possibly due to impaired expression of *sek-1* by disrupting the intron between the exons encoding S204D and T208D.

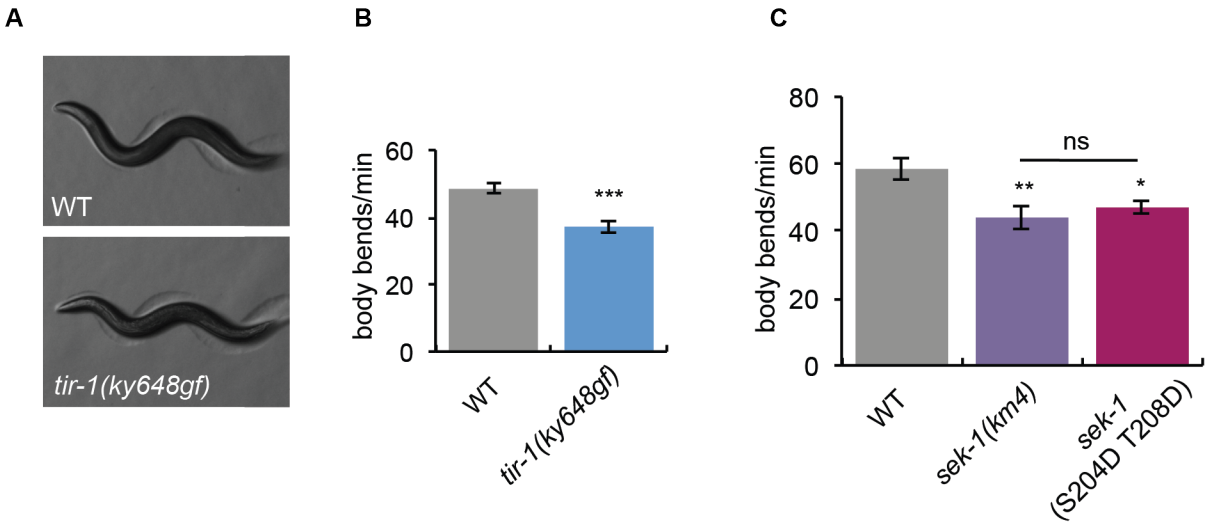

Figure 2.6: Activating the p38 MAPK pathway results in locomotion defects
(A-B) *tir-1(ky648gf)* animals do not have loopy or hyperactive locomotion. *tir-1(ky648gf)* worms have wild-type posture and are slower than wild-type animals. ***, p< 0.001, error bars = SEM, n=20.
(C) *sek-1*(S204D T208D) animals have slow locomotion. Worms with the S204D T208D variant of *sek-1* move slower than wild-type and do not move faster than *sek-1(km4)* animals. **, p< 0.01; *, p<0.05, error bars = SEM, n=10.

Genetic interactions of *sek-1* with pathways acting downstream of Gq

Our forward genetic screen for suppressors of *egl-30(gf)* identified mutants that fall into three different categories: mutants in the canonical Gq pathway such as the PLC *egl-8* (Lackner et al., 1999), mutants in the RhoGEF Trio pathway such as *unc-73* (Williams et al., 2007), and mutants that affect dense-core vesicle biogenesis and release (Ailion et al., 2014; Topalidou et al., 2016a).

To test if *sek-1* acts in these pathways we used genetic epistasis analysis. Loss-of-function alleles of *egl-8(sa47)*, *unc-73(ox317),* and *rund-1(tm3622)* have slow locomotion (Figure 2.7 A-C). Our data show that *sek-1* enhances the phenotype of *egl-8* and *rund-1* single mutants, suggesting that *sek-1* does not act in the same pathway as *egl-8* or *rund-1* (Figure 2.7 A, B). By contrast, *sek-1* does not enhance the slow

locomotion phenotype of *unc-73* mutants (Figure 2.7 C), suggesting that *sek-1* may act in the same genetic pathway as the Trio RhoGEF *unc-73.*

We also tested whether *sek-1* interacts with *rho-1*, encoding the small G protein activated by Trio. *rho-1* is required for viability so we could not use a loss-of-function allele to test for a genetic interaction (Jantsch-Plunger et al., 2000). Instead, we used an integrated transgene overexpressing an activated *rho-1* mutant allele specifically in cholinergic neurons. Animals carrying this activated RHO-1 transgene, referred to here as *rho-1(gf)*, have a loopy posture reminiscent of *egl-30(gf)* (McMullan et al., 2006), and a decreased locomotion rate. *rho-1(gf) sek-1(km4)* double mutants had a loopy body posture like *rho-1(gf)* and an even slower locomotion rate (Figure 2.7 D, E), suggesting that *sek-1* and *rho-1(gf)* mutants have additive locomotion phenotypes. However, both *sek-1(km4)* and *sek-1(yak42)* suppress the slow growth rate of the *rho-1(gf)* mutant. Because *sek-1* does not enhance *unc-73* mutants and suppresses some aspects of the *rho-1(gf)* mutant, *sek-1* may modulate output of the Rho pathway, though it probably is not a direct transducer of Rho signaling.

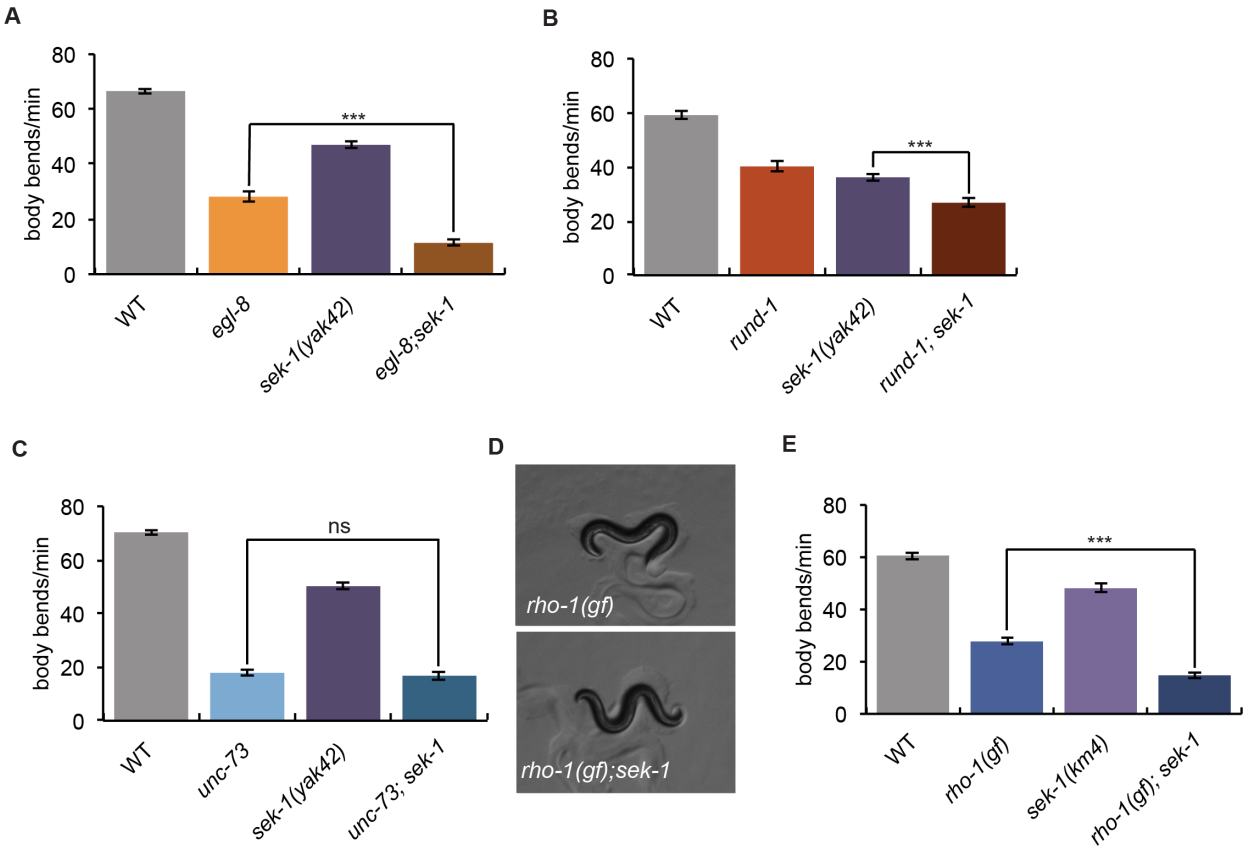

Figure 2.7: *sek-1* acts in the same genetic pathway as *unc-73*
(A) *sek-1* does not act in the same genetic pathway as *egl-8.* The *sek-1(km4)* mutation enhances the slow locomotion of *egl-8(sa47)* mutants. ***, p< 0.001, error bars = SEM, n=20.
(B) *sek-1* does not act in the same genetic pathway as *rund-1*. The *sek-1(km4)* mutation enhances the slow locomotion of *rund-1(tm3622)* mutants. ***, p< 0.001, error bars = SEM, n=20.
(C) *sek-1* may act in the same genetic pathway as *unc-73*. The *sek-1(km4)* mutation does not enhance the slow locomotion phenotype of *unc-73* mutants. ns, p>0.05, error bars = SEM, n=20.
(D) *sek-1(km4)* does not suppress the high-amplitude waveform of *nzIs29 rho-1(gf)* animals. *rho-1(gf)* and *rho-1(gf) sek-1(km4)* animals have similar body posture.
(E) *sek-1(km4)* does not suppress the slow locomotion of *rho-1(gf)* animals. ***, p< 0.001, error bars = SEM, n=20.

sek-1 and *nsy-1* partially suppress activated NCA

The data above do not clarify the relationship of *sek-1* to the Rho pathway acting downstream of Gq. The downstream target of this Gq-Rho pathway appears to be the

NCA-1 cation channel (Topalidou et al., 2016b). NCA-1 and its orthologs are sodium leak channels associated with rhythmic behaviors in several organisms (Nash et al., 2002; Lu et al., 2007; Shi et al., 2016). In *C. elegans*, NCA-1 potentiates persistent motor circuit activity and sustains locomotion (Gao et al., 2015).

To examine interactions of the *sek-1* p38 MAPK pathway with NCA-1, we tested whether *sek-1* and *nsy-1* mutants suppress the activated NCA-1 mutant *ox352*, referred to as *nca-1(gf)*. The *nca-1(gf)* animals are coiled and uncoordinated; thus, it is difficult to measure their locomotion rate by the body-bend assay because they sometimes do not propagate sinusoidal waves down the entire length of their body. Instead, we used a radial locomotion assay in which we placed animals in the center of a 10-cm plate and later measured how far the animals had moved. *nca-1(gf)* double mutants with either *sek-1(km4)* or *nsy-1(ok593)* uncoil a bit but still exhibit uncoordinated locomotion (Figure 2.8A). In fact, though these double mutants show more movement in the anterior half of their bodies than *nca-1(gf)*, they propagate body waves to their posterior half even more poorly than the *nca-1(gf)* mutant. However, both *sek-1* and *nsy-1* clearly suppress the small size and slow growth rate of the *nca-1(gf)* mutant (Figure 2.8 A) and in radial locomotion assays, *sek-1* and *nsy-1* weakly suppressed the *nca-1(gf)* locomotion phenotype (Figure 2.8B). Together these data suggest that mutants in the *sek-1* p38 MAPK pathway partially suppress some aspects of the *nca-1(gf)* mutant.

Given that *sek-1* acts in cholinergic neurons to regulate wild-type and *egl-30(gf)* locomotion, we tested if the same neuron class is responsible for *sek-1* suppression of *nca-1(gf)*. Expression of *sek-1* in all neurons or in cholinergic neurons of *nca-1(gf) sek-1(km4)* animals restored the *nca-1(gf)* size and posture phenotypes. These worms are

small and coiled and closely resemble the *nca-1(gf)* mutant (Figure 2.8C). By contrast, expression of *sek-1* in GABA neurons did not affect the size or posture of the *nca-1(gf) sek-1* double mutant (Figure 2.8C). These data suggest that *sek-1* acts in cholinergic neurons to regulate *nca-1(gf)* locomotion. However, in radial locomotion assays, expression of *sek-1* in none of these neuron classes significantly altered the movement of the *nca-1(gf) sek-1* double mutant (Figure 2.8D), though the weak suppression of *nca-1(gf)* by *sek-1* in this assay makes it difficult to interpret these negative results. We make the tentative conclusion that *sek-1* partially suppresses *nca-1(gf)* locomotion and probably acts in the cholinergic neurons where it also acts to regulate wild-type and *egl-30(gf)* locomotion, suggesting a common neuronal site of action of this pathway.

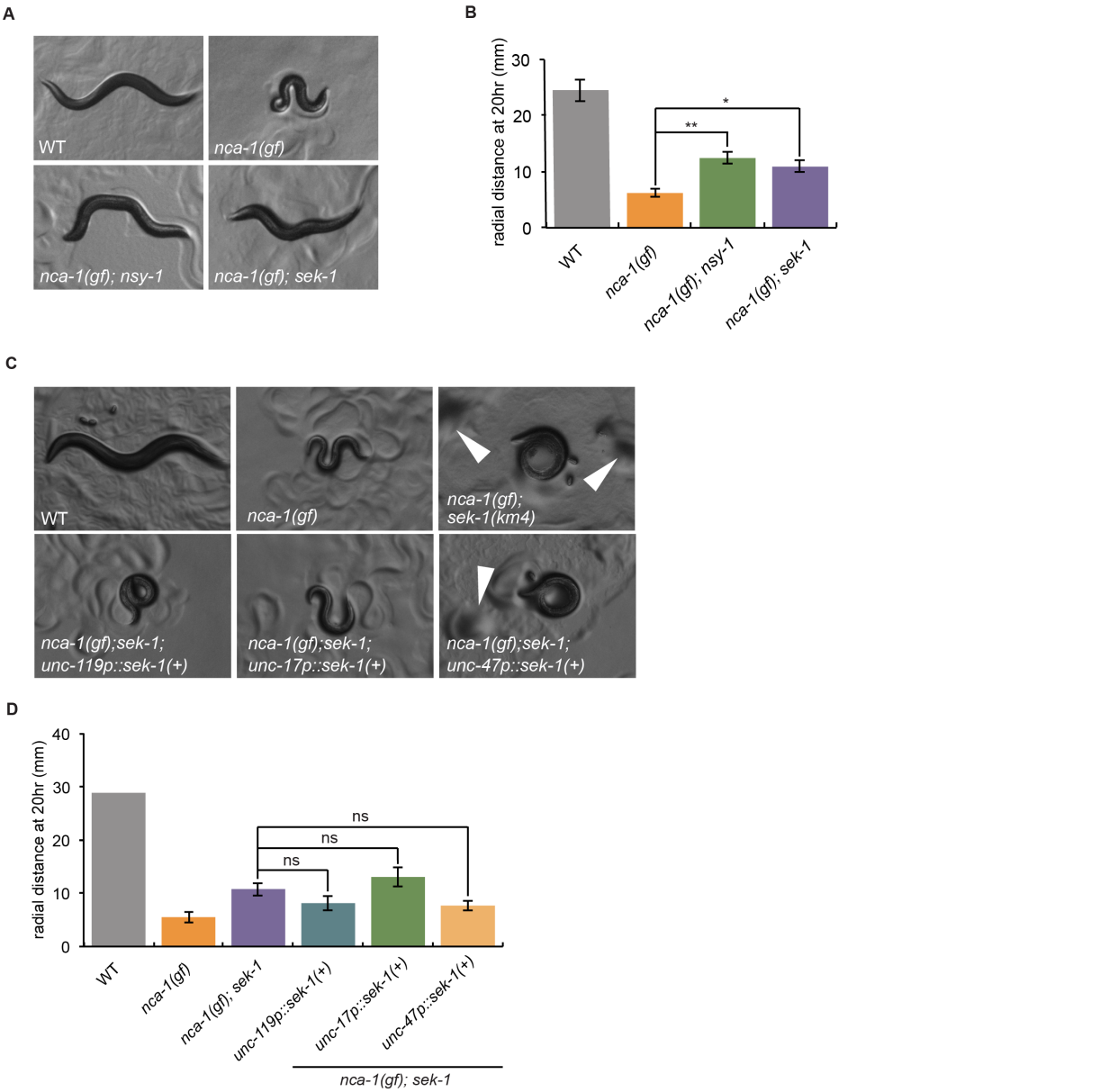

Figure 2.8: *sek-1* and *nsy-1* weakly suppress *nca-1(gf)*
(A) *nca-1(gf)* mutants are small and tightly coiled. The phenotypes of these *nca-1(ox352)* animals are partially suppressed by *nsy-1(ok593)* and *sek-1(km4)*. Photos of first-day adults.
(B) *nsy-1* and *sek-1* suppress *nca-1(gf)* locomotion. *nca-1(gf)* animals travel a small distance from the center of the plate in the radial locomotion assay. *nca-1(gf) nsy-1(ok593)* and *nca-1(gf) sek-1(km4)* worms move further than *nca-1(gf)* worms. **, $p<0.01$; *, $p<0.05$. Error bars = SEM, n=30.
(C) Expression of *sek-1* in all neurons and in cholinergic neurons partially reverts the *sek-1* mutant suppression of *nca-1(gf)* size and body posture. White arrowheads depict food piles created by *nca-1(gf) sek-1(km4)* animals due to their uncoordinated locomotion. Such food piles are not made by *nca-1(gf)* animals.

(D) None of the neuronal *sek-1* rescuing constructs revert the radial locomotion phenotype of *nca-1(gf) sek-1(km4)* animals. Although the pan-neuronal and cholinergic neuron constructs revert the size phenotype of *nca-1(gf) sek-1(km4)*, these animals do not move a significantly different distance than *nca-1(gf) sek-1(km4)* animals. ns, $p>0.05$. Error bars = SEM, n=19-24.

Discussion

In this study we identified a new neuronal role for the mitogen-activated protein kinase kinase SEK-1 and the p38 MAPK pathway as a positive regulator of locomotion and Gq signaling. The p38 MAPK pathway has been best characterized as a pathway activated by a variety of cellular stresses and inflammatory cytokines (Kyriakis and Avruch, 2012), but it has also been implicated in neuronal function, including some forms of mammalian synaptic plasticity (Bolshakov et al., 2000; Huang et al., 2004; Rush et al., 2002). In *C. elegans*, Gq plays a positive role in locomotion by promoting acetylcholine release, so we tested whether the slow locomotion of *sek-1* mutants is directly related to the role of *sek-1* as a regulator of Gq signaling. Through rescue experiments, we found that SEK-1 acts in cholinergic neurons to regulate both the rate of locomotion and Gq signaling. Previously, *sek-1* has been shown to act in GABA neurons to regulate sensitivity to the acetylcholinesterase inhibitor aldicarb (Vashlishan et al., 2008), and to act in interneurons to regulate trafficking of the GLR-1 glutamate receptor and the frequency of worm reversals (Park and Rongo, 2016). Thus, *sek-1* may play distinct roles in different neuron types. Our data indicate a role for *sek-1* as a positive regulator of Gq signaling in cholinergic neurons.

In addition to SEK-1, we identified other p38 pathway components regulating Gq signaling. Specifically, we found that mutants in *tir-1*, *nsy-1* and a *pmk-1 pmk-2* double mutant exhibit locomotion defects identical to *sek-1* and suppress activated Gq,

suggesting that they act in a linear p38 pathway to modulate signaling downstream of Gq. These results indicate a redundant function for PMK-1 and PMK-2 in regulating locomotion rate and Gq signaling. PMK-1 and PMK-2 also act redundantly for development of the asymmetric AWC neurons and to regulate induction of serotonin biosynthesis in the ADF neurons in response to pathogenic bacteria (Pagano et al., 2015). By contrast, PMK-1 acts alone in the intestine to regulate innate immunity and in interneurons to regulate GLR-1 trafficking (Pagano et al., 2015; Park and Rongo, 2016).

Activation of the p38 MAPK signaling module with *tir-1(ky648gf)* or *sek-1*(S204D T208D) did not cause any phenotypes similar to *egl-30(gf)*. Recently, a *nsy-1(gf)* allele was isolated that also causes slow development and reduced brood sizes (Cheesman et al., 2016). It is possible that p38 pathway activation in other tissues or neuron types is masking possible phenotypes. Future studies can explore whether expressing *(gf)* constructs in cholinergic neurons may confer gain-of-function locomotion phenotypes.

What is the downstream effector of *sek-1* p38 MAPK signaling in this Gq signaling pathway? There are several known downstream effectors of p38 MAPK signaling in *C. elegans*, including the transcription factor ATF-7 (Shivers et al., 2010; Inoue et al., 2005; Xie et al., 2013). Our data indicate that ATF-7 is not required for the p38 MAPK-dependent regulation of Gq signaling. It is possible that this p38 MAPK pathway activates molecules other than transcription factors to regulate Gq signaling. It is also possible that the *sek-1* p38 pathway activates multiple downstream effectors that are not individually required.

One of the pathways that transduce signals from Gq includes the RhoGEF Trio/UNC-73, the small GTPase Rho, and the cation channel NALCN/NCA-1 (Williams

et al., 2007; Topalidou et al., 2016b). We found that mutations in the *sek-1* p38 MAPK pathway partially suppress an activated NCA-1 mutant and that *sek-1* probably acts to control NCA-1 activity in the same neurons where it acts to regulate locomotion and Gq signaling. Given the precedence for direct phosphorylation of sodium channels by p38 to regulate channel properties (Wittmack et al., 2005; Hudmon et al., 2008), it is possible that PMK-1 and PMK-2 phosphorylate NCA-1 to regulate its expression, localization, or activity. Our genetic study sets the foundation for further investigation of the specific role of the *sek-1* p38 pathway in the regulation of Gq signaling and NCA-1 channel activity.

Materials and Methods

C. elegans strains and maintenance

All strains were cultured using standard methods and maintained at 20°C (Brenner, 1974). The *sek-1(yak42)* mutant was isolated from an ENU mutagenesis suppressor screen of the activated Gq mutant *egl-30(tg26)* ("*egl-30(gf)*") (Ailion et al., 2014). *sek-1(yak42)* was outcrossed away from *egl-30(gf)* before further analysis. Double mutant strains were constructed using standard methods (Fay, 2006) often with linked fluorescent markers (Frokjaer-Jensen et al., 2014) to balance mutations with subtle visible phenotypes. Appendix 1 contains all the strains used in this study.

Mapping

yak42 was mapped using the slow locomotion phenotype and its *egl-30(gf)* suppression phenotype. *yak42* was initially mapped to chromosome X using strains

EG1000 and EG1020, which carry visible marker mutations. These experiments showed that *yak42* was linked to *lon-2*, but at least several map units away. *yak42* was further mapped to about one map unit (m.u.) away from the red fluorescent insertion marker *oxTi668* which is located at +0.19 m.u. on Chromosome X.

Molecular biology

Plasmids were constructed using the Gateway cloning system (Invitrogen). Plasmids used are found in Appendix 3. The *sek-1* cDNA was amplified by RT-PCR from worm RNA and cloned into a Gateway entry vector. To check for proper expression of *sek-1*, an operon GFP was included in expression constructs with the following template: (promoter)p::*sek-1*(cDNA)::*tbb-2utr::gpd-2 operon::GFP::H2B:cye-1utr* (Frøkjær-Jensen et al., 2012). This resulted in untagged SEK-1, but expression could be monitored by GFP expression.

Injections

C. elegans strains with extrachromosomal arrays were generated by standard methods (Mello et al., 1991). Injection mixes were made with a final total concentration of 100 ng/µL DNA. Constructs were injected at 5 ng/µL, injection markers at 5 ng/µL, and the carrier DNA Litmus 38i at 90 ng/µL. Multiple lines of animals carrying extrachromosomal arrays were isolated and had similar behaviors as observed by eye. The line with the highest transmittance of the array was assayed.

CRISPR/Cas9 genome editing

CRISPR/Cas9 genome editing was performed as described by Dickinson, et al. 2013. 10 ng/uL homology-repair template, 50 ng/uL sgRNA-Cas9 plasmid, and co-injection markers were injected into EG6207 *unc-119(ed3)* worms.

Whole-genome sequencing

Strain XZ1233 *egl-30(tg26); yak42* was used for whole-genome sequencing to identify candidate *yak42* mutations. XZ1233 was constructed by crossing a 2X outcrossed *yak42* strain back to *egl-30(tg26)*. Thus, in XZ1233, *yak42* has been outcrossed 3X from its original isolate. DNA was isolated from XZ1233 and purified according to the Hobert Lab protocol (http://hobertlab.org/whole-genome-sequencing/). Ion Torrent sequencing was performed at the University of Utah DNA Sequencing Core Facility. The resulting data contained 10,063,209 reads of a mean read length of 144 bases, resulting in about 14X average coverage of the *C. elegans* genome. The sequencing data were uploaded to the Galaxy web platform and we used the public server at usegalaxy.org to analyze the data (Afgan et al., 2016). We identified and annotated variants with the Unified Genotyper and SnpEff tools, respectively (Cingolani et al., 2012; DePristo et al., 2011). We filtered out variants found in other strains we sequenced, leaving us with 605 homozygous mutations. A detailed protocol for WGS data analysis is in Appendix 2. Chromosome X contained 94 mutations: 55 SNPs and 39 indels. Of these, four SNPs were non-synonymous mutations in protein-coding genes, but only 2 were within 5 m.u. of *oxTi668.* However, we were unable to identify *yak42* from the candidate polymorphisms located near *oxTi668*. Transgenic expression

of the most promising candidate *pcyt-1* (located at -1.49 m.u.) did not rescue *yak42*. Instead, to identify possible deletions, we scrolled through 2 MB of aligned reads on the UCSC Genome Browser starting at -4.38 m.u. and worked towards the middle of the chromosome (0 m.u.), looking for regions that lacked sequence coverage. We found a 3713 bp deletion that was subsequently confirmed to be the *yak42* causal mutation, affecting the gene *sek-1* located at -1.14 m.u.

Locomotion assays

Locomotion-assay plates were made by seeding 10-cm nematode growth medium plates with 150 µl of an OP50 (a strain of *E. coli*) stock culture, spread with sterile glass beads to cover the entire plate. Bacterial lawns were grown at room temperature (22.5°C – 24.5°C) for 24 hrs and then stored at 4°C until needed. All locomotion assays were performed on first-day adults. L4-stage larvae were picked the day before the assay and the experimenter was blind to the genotypes of the strains assayed. For experiments on strains carrying extrachromosomal arrays, the *sek-1(km4)* control worms were animals that had lost the array from the same plate.

Body-bends assays were performed as described (Miller et al., 1999). The locomotion-assay plate was brought to room temperature (22.5°C – 24.5°C). All strains in an experiment were assayed on the same assay plate. A single animal was picked onto the plate, the plate lid was returned, and the animal was allowed recovered for 30 s. Body bends were then counted for one minute. A body bend was counted each time the worm's tail reached the minimum or maximum amplitude of the sine wave. For experiments with *egl-8*, *unc-73*, and *rund-1*, worms were allowed a minimal recovery

period (until the worms started moving forward, 5 seconds maximum) prior to counting body bends.

For the heat-shock experiment, plates of first-day adults were parafilmed and heat-shocked in a 34°C water bath for 1 hr. Plates were then un-parafilmed and incubated at 20°C for five hours before performing body-bend assays.

Radial locomotion assays were performed by first bringing the locomotion-assay plates to room temperature. Animals were picked to the middle of the plate, the plate taped shut, and an x was marked on the lid above where the animals were placed. Assay plates were then incubated at 20°C for 20 hr and the distances of the worms from the starting point were measured.

Quantitative analysis of the waveform of worm tracks was performed by first bringing the locomotion-assay plate to room temperature. One strain at a time, five animals were placed on the plate and allowed to roam for 2 – 5 min. We then took five pictures of each animal's tracks following forward locomotion. Track pictures were taken at 40X on a Nikon SMZ18 microscope with the DS-L3 camera control system. This process was repeated for all strains per experiment set. Worm-track pictures were processed using ImageJ. Tracks were straightened with the segmented line tool and pictures converted to grayscale. Period and 2X amplitude were measured freehand using the line tool. For each worm, five period/amplitude ratios were calculated from each of the five straightened tracks. The ratios were averaged for each worm and these averages were averaged for each strain.

C. elegans pictures

Pictures of worms were taken at 60X on a Nikon SMZ18 microscope with the DS-L3 camera control system. The worms were age-matched as first day adults and each experiment set was photographed on the same locomotion assay plate (assay plate preparation described above). The images were processed using ImageJ and were rotated, cropped, and converted to grayscale.

Statistical analysis

At the beginning of the project, a power study was conducted on pilot body-bend assays using wild-type and *sek-1(yak42)* worms. To achieve a power of 0.95, it was calculated that 17 animals should be assayed per experiment. Data were analyzed to check if normally distributed (using the D'Agostino-Pearson and Shapiro-Wilk normality tests) and then subjected to the appropriate analysis using GraphPad Prism 5. For data sets with three or more groups, if the data were normal they were analyzed with a one-way ANOVA; if not, with a Kruskal-Wallis test. Post-hoc tests were used to compare appropriate data sets within an experiment. Reported p-values are corrected. $p<0.05$ = *; $p<0.01$ = **; $p<0.001$ = ***.

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Chapter 3

Conclusions and Future Directions

Conclusions

In this study we identified a new neuronal role for SEK-1 and the p38 MAPK pathway as a positive regulator of locomotion and Gq signaling (Figure 3.1). We found that mutations in *sek-1* are suppressors of activated Gq (*egl-30(gf)* in *C. elegans*). We studied different *sek-1* mutants and found that they had slow locomotion compared to wild-type worms. We found that *sek-1* acts in cholinergic neurons to regulate locomotion and Gq signaling.

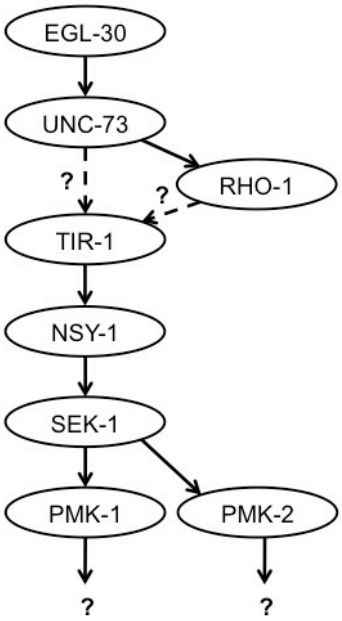

Figure 3.1: Possible pathway of how Gq/EGL-30 signals to TIR-1 and the p38 MAPK pathway. Solid arrows are known interactions and dashed arrows are unknown interactions.

SEK-1 is a MAPKK so we investigated if SEK-1 acts as a part of a MAPK pathway to regulate Gq signaling and locomotion. SEK-1 functions downstream of NSY-1 (a MAPKKK) and we found that worms with mutant *nsy-1* exhibit the same phenotypes as *sek-1*. We also found that the *C. elegans* p38 MAPK orthologs *pmk-1* and *pmk-2* function redundantly in their involvement in Gq signaling and regulation of locomotion, because the single mutants displayed no locomotion phenotype or Gq suppression but the double mutants had the same phenotypes as *sek-1* and *nsy-1*. Furthermore, we also identified TIR-1, a signaling component shown to function upstream of NSY-1, as a

regulator of Gq signaling and locomotion. We conclude that the TIR-1/NSY-1/SEK-1/PMK-1 PMK-2 signaling module functions as a pathway to regulate locomotion and Gq signaling.

SEK-1 acts in the same pathway as UNC-73, which is the GEF for RHO-1. We studied worms with *rho-1(gf)* (which is only expressed in cholinergic neurons) to see if *sek-1* affected its phenotypes. Our results suggest that SEK-1 likely doesn't act downstream of RHO-1 in a clear pathway but may modulate some RHO-1 signals. Another protein known to act downstream of Gq and UNC-73 is an ion channel, NCA-1. We found that *nsy-1* and *sek-1* partially suppress *nca-1(gf)* which suggests that the p38 MAPK pathway affects NCA-1.

Future studies can fill out this Gq signaling pathway and explore the effects of the p38 MAPK pathway in Gq signaling and locomotion.

Future Directions

How does Gq signal to SEK-1? While mutations in the p38 MAPK pathway clearly suppress *egl-30(gf)*, the connection from Gq to this pathway is not clear (Figure 3.1). The two known signaling pathways downstream of Gq in *C. elegans* are through EGL-8 (PLCβ) and UNC-73 (Trio RhoGEF). Through our genetic experiments, we saw that *sek-1* acts in the same pathway as *unc-73*. UNC-73 activates RHO-1, but our data suggest that the p38 MAPK is not a direct transducer of RHO-1 signaling.

If the TIR-1/NSY-1/SEK-1/PMK-1 PMK-2 signaling module acts in the same cell as Gq to regulate Gq signaling, it would presumably act downstream of UNC-73. The

p38 MAPK signaling module that acts downstream of Gq starts with TIR-1. If we could identify proteins that interact with TIR-1, we could potentially bridge the gap between UNC-73 and TIR-1. Alternatively, UNC-73 could directly interact with TIR-1. We can test if UNC-73 interacts directly with TIR-1 by expressing tagged versions of both proteins and performing a Co-IP experiment to see if they interact. There are currently no reports of TIR-1 (or its ortholog SARM) interacting with a RhoGEF.

TIR-1 has several known interactors such as CamKII/UNC-43 (Chuang and Bargmann, 2005) and the GTPase RAB-1 (Couillault et al., 2004) that we can investigate as possible intermediates between TIR-1 and UNC-73. Another way to find TIR-1 interactors is to do a pull down experiment and identify interactors using mass spectrometry, which our lab has done before for identifying interactors of proteins such as EIPR-1 (Topalidou et al., 2016). By identifying TIR-1 interactors, we could learn how Gq signaling is transduced through UNC-73 to the p38 MAPK signaling module.

Where does *sek-1* act? Our study shows that *sek-1* acts in cholinergic neurons to regulate locomotion and Gq signaling. There are sub-classes of cholinergic neurons, such as those in the head of the animal and the body. We have done some preliminary experiments testing which cholinergic promoter is sufficient to rescue the *sek-1* phenotypes. We expressed *sek-1* under the head-cholinergic-neuron promoter and found that it did not rescue the *sek-1* slow locomotion phenotype, but it did revert the *egl-30(gf)* suppression (Table 3.1). However, *sek-1* expression using a body-cholinergic-neuron promoter rescued the locomotion phenotype, but not the *egl-30(gf)* suppression. These results suggest that *sek-1* could act in different neurons to

transduce Gq signals (in the head-cholinergic neurons) and regulate locomotion (in the body-cholinergic neurons). Our study also showed that *sek-1* partially suppresses *nca-1(gf)* and preliminary results suggest that this suppression is through the cholinergic body promoter. We will perform quantitative experiments on these worms to explore the site of *sek-1* actions for its role in regulating locomotion and suppressing *egl-30(gf)* and *nca-1(gf)*.

Table 3.1 *sek-1* acts in different neurons to rescue different phenotypes

	Expression of *sek-1* in…	
background	head cholinergic neurons	body cholinergic neurons
sek-1(km4)	no rescue	rescue
egl-30(gf);sek-1(km4)	rescue	no rescue
nca-1(gf);sek-1(km4)	no rescue	rescue

How does the p38 MAPK pathway affect NCA-1? Mutations in *sek-1* and *nsy-1* partially suppress *nca-1(gf)*, so we conclude that the p38 MAPK pathway affects NCA-1. This pathway could affect NCA-1 in many ways such as changing its expression, localization, or activity.

We can explore whether the p38 pathway affects NCA-1 expression and localization in many ways. We have integrated transgenes of NCA-1::GFP and strains containing this transgene with the *nsy-1* mutation. Preliminary experiments show no difference of total fluorescence between NCA-1::GFP and *nsy*-1;NCA-1::GFP but a more careful study may reveal subtle differences in localization of NCA-1::GFP. We can also use RT-qPCR to examine expression levels of *nca-1* in the *sek-1, nsy-1* or *pmk-1 pmk-2* p38 mutant background.

NCA-1 does not function alone. NCA-1 has auxiliary subunits such as UNC-79 and UNC-80 (Yeh et al., 2008) so we can also explore if the p38 MAPK pathway affects

their expression by RT-qPCR. NCA-1 is one of two NALCN (a sodium leak channel) homologs in *C. elegans*. The other NALCN ortholog in *C. elegans,* NCA-2, functions redundantly with NCA-1 to affect locomotion and synaptic activity (Pierce-Shimomura et al., 2008; Yeh et al., 2008). We already have strains with an integrated NCA-2::GFP and already have made *nsy-1;*NCA-2::GFP. We can use these strains to see if the p38 MAPK pathway affects the localization or expression of NCA-2::GFP. These different techniques can test if and how the p38 MAPK pathway affects NCA-1, NCA-2, UNC-80, and UNC-79 expression or localization and may shed light on how this pathway is connected to NCA-1.

It is also possible to test how and if the p38 MAPK pathway affects the activity of NCA-1 and NCA-2. NCA-1 and NCA-2 act redundantly and are required for rhythmic bursts of postsynaptic currents (rPSCs) in *C. elegans* (Gao et al., 2015). We can test if the p38 MAPK pathway affects NCA activity by measuring rPSCs in worms with a wild-type background versus worms with a *sek-1, nsy-1* or *pmk-2 pmk-1* mutant background. If the p38 MAPK pathway affects NCA activity, there should be fewer rPSCs.

What does the p38 MAPK pathway target? The p38 MAPK pathway has many possible targets. MAPKs phosphorylate their targets so it would be useful to see what proteins are phosphorylated or not phosphorylated in the absence of PMK-1 and PMK-2. For this study we would compare the phosphoproteomes of wild-type worms and *pmk-1 pmk-2* worms. It is likely that the phosphoproteomes will have many differences and we will focus our attention on proteins known to act in neurons and signal transduction proteins.

It is possible we have already isolated mutations in components downstream of the p38 MAPK pathway from our forward genetic screen on *egl-30(gf)*. Many of the suppressors we isolated are currently unidentified. The lab already has sequencing data on several *egl-30(gf)* suppressor mutations, so we can use these data to identify the causal suppressor mutation. Like we did in this study, we can place suppressors in different pathways downstream of Gq with genetic epistasis analyses. We have many other Gq suppressor strains that we can send to whole genome sequencing and analyze that resulting data (as detailed in Appendix 3).

Do *nsy-1(gf)* or *sek-1(gf)* cause locomotion phenotypes? It is possible to make gain-of-function alleles of *nsy-1* and *sek-1*. Deleting the first 640 animo acids of NSY-1 causes a gain-of-function phenotype in AWC neuron specification (Sagasti et al., 2001). Recently, a gain-of-function *nsy-1* allele (*ums8*) was isolated that confers a gain-of-function phenotype in a *C. elegans* innate immunity study (Cheesman et al., 2016). The *ums8* allele causes R246Q and worms with *nsy-1(ums8)* develop slower than wild-type worms and have smaller brood sizes.

NSY-1 phosphorylates SEK-1 on S204 and T208 and mimicking these phosphorylation events causes a gain-of-function phenotype in AWC specification (Tanaka-Hino et al., 2002). We attempted to make a *sek-1(gf)* allele using CRISPR/Cas9 but it had a loss-of-function phenotypes for AWC neuron specification and locomotion. It is possible that the CRISPR/Cas9 genome editing disturbed *sek-1* expression. S204 and T208 are encoded by adjacent exons and our genome editing strategy included replacing the intron between these exons with a selectable marker.

This new intron may have disrupted *sek-1* expression. Our study also found that *tir-1(gf)* didn't have a locomotion phenotype reminiscent of *egl-30(gf)*.

The p38 MAPK pathway functions in different tissues, such as neurons and the intestine. The *tir-1(gf)* and *nsy-1(ums8*) alleles affect all *tir-1* and *nsy-1* transcripts (respectively). Our study shows that SEK-1 functions in cholinergic neurons to regulate locomotion and Gq signaling. To test the effect of the p38 MAPK in neurons, we can express gain-of-function constructs under neuronal promoters and study the resulting phenotypes. Previous studies used this approach to see *nsy-1(gf)* and *sek-1(gf)* phenotypes in AWC neuron specification (Sagasti et al., 2001; Tanaka-Hino et al., 2002). We can create constructs using *nsy-1* and *sek-1* cDNA with the mutations that cause the gain-of-function phenotypes. We can put these mutated cDNAs under different neuronal promoters to express *sek-1(gf)* and *nsy-1(gf)* and study worms that express these constructs for locomotion phenotypes. To avoid artifacts of grossly overexpressing transgenes, we can use the MosSCI method to create single copy insertions of these constructs (Frokjaer-Jensen et al., 2014). If worms expressing these constructs have a phenotype like the hyperactive, loopy phenotype of *egl-30(gf)* worms, we can use the *sek-1(gf)* or *nsy-1(gf)* worms for a forward genetic screen. Screening for suppressors of *nsy-1(gf)* or *sek-1(gf)* could identify loss-of-function alleles in genes that are downstream of the p38 MAPK pathway.

Appendix 1

C. elegans Strain List

Strain	Genotype
AU1	*sek-1(ag1)* X
BS3383	*pmk-3(ok169)* IV
CX3695	*kyIs140[str-2p::gfp, lin-15(+)]* I
CX5959	*kyIs140[str-2p::gfp, lin-15(+)]* I; *tir-1(ky648gf)* III
EG317	*unc-73(ox317)* I
EG1000	*dpy-5(e61)* I; *rol-6(e187)* II; *lon-1(e1820)* III
EG1020	*bli-6(sc16)* IV; *dpy-11(e224)* V; *lon-2(e678)* X
EG4782	*nzIs29[unc-17p::rho-1(G14V), unc-122::gfp]* II
EG5505	*rund-1(tm3622)* X
EG7989	*unc-119(ed3)* III; *oxTi668[eft-3p::TdTomato::H2B, Cb-unc-119(+)]* X
IG685	*tir-1(tm3036)* III
JN147	*gap-2(tm748)* X
JT47	*egl-8(sa47)* V
JT366	*vhp-1(sa366)* II
JT734	*goa-1(sa734)* I
KU2	*jkk-1(km2)* X
KU4	*sek-1(km4)* X
KU25	*pmk-1(km25)* IV
N2	Bristol wild isolate, standard lab wild-type
VC8	*jnk-1(gk7)* IV
VC390	*nsy-1(ok593)* IV
XZ42	*sek-1(yak42)* X
XZ1233	*egl-30(tg26)* I*; sek-1(yak42)* X
XZ1151	*egl-30(tg26)* I
XZ1566	*egl-8(sa47)* V*; sek-1(yak42)* X
XZ1567	*unc-73(ox317)* I*; sek-1(yak42)* X
XZ1574	*rund-1(tm3622) sek-1(yak42)* X
XZ1575	*egl-30(tg26)* I*; sek-1(km4)* X
XZ1588	*egl-30(tg26)* I*; nsy-1(ok593)* IV
XZ1589	*egl-30(tg26)* I*; sek-1(km4)* X*; qdEx8[unc-119p::sek-1::GFP, myo-2p::mStrawberry::unc-54-3'UTR]*
XZ1593	*egl-30(tg26)* I*; pmk-1(km25)* IV
XZ1642	*sek-1(km4)* X*; yakEx72[unc-17p::sek-1::tbb-2utr-operon-GFP::H2B::cye-1utr, myo-2p::mCherry]*
XZ1643	*sek-1(km4)* X*; yakEx73[unc-47p::sek-1::tbb-2utr-operon-GFP::H2B::cye-1utr, myo-2p::mCherry]*
XZ1651	*unc-119(ed3)* III; *sek-1(yak115*[S204D T208D *unc-119(+)*]*)* X

XZ1717	*nzIs29[unc-17p::rho-1(G14V) unc-122::gfp]* II*; sek-1(km4)* X
XZ1770	*egl-30(tg26)* I*; pmk-2(qd279 qd171) pmk-1(km25)* IV
XZ1771	*egl-30(tg26)* I*; pmk-2(qd287)* IV
XZ1772	*egl-30(tg26)* I*; pmk-3(ok169)* IV
XZ1815	*egl-30(tg26)* I*; tir-1(tm3036)* III
XZ1816	*nca-1(ox352)* IV*; sek-1(km4)* X*; qdEx8[unc-119p::sek-1::GFP::unc-54-3' UTR, myo-2p::mStrawberry::unc-54-3'UTR]*
XZ1834	*egl-30(tg26)* I*; sek-1(km4)* X*; yakEx72[unc-17p::sek-1::tbb-2utr-operon-GFP::H2B::cye-1utr, myo-2p::mCherry]*
XZ1835	*nca-1(ox352)* IV*; sek-1(km4)* X*; yakEx72[unc-17p::sek-1::tbb-2utr-operon-GFP::H2B::cye-1utr, myo-2p::mCherry]*
XZ1861	*nca-1(ox352)* IV*; sek-1(km4)* X*; yakEx73[unc-47p::sek-1::tbb-2utr-operon-GFP::H2B::cye-1utr, myo-2p::mCherry]*
XZ1863	*egl-30(tg26)* I*; sek-1(km4)* X*; yakEx73[unc-47p::sek-1::tbb-2utr-operon-GFP::H2B::cye-1utr, myo-2p::mCherry]*
XZ1872	*jnk-1(gk7)* I*; sek-1(km4)* X
XZ1873	*pmk-2(qd279 qd171) pmk-1(km25)* IV*; jkk-1(km2)* X
XZ1937	*sek-1(km4)* X*; yakEx121[hsp-16.2p::sek-1::tbb-2-3' UTR::gld-1 operon linker::gfp::h2b, myo-2p::mCherry]*
XZ1939	*goa-1(sa734)* I*; sek-1(km4)* X
XZ1942	*tir-1(ky648gf)* III
ZD202	*sek-1(km4)* X*; qdEx8[unc-119p::sek-1::GFP::unc-54-3' UTR + myo-2p::mStrawberry::unc-54-3'UTR]*
ZD318	*agIs29 atf-7(qd22 qd130)* III
ZD442	*agIs29 atf-7(qd22)* III
ZD934	*pmk-2(qd279 qd171) pmk-1(km25)* IV
ZD1020	*pmk-2(qd287)* IV

Appendix 2

List of Plasmids

Gateway entry clones

Plasmid	Details
pJH19	*sek-1* homology right arm [2-3]
pJH20	*sek-1* homology left arm [1-4]
pJH21	*sek-1* cDNA [1-2]
pCFJ150	pDEST5605[4-3]
pCFJ326	*tbb-2utr-operon-GFP::H2B::cye-1utr* [2-3]
pMH522	*unc-47p* [4-1]
pGH1	*unc-17p* [4-1]
pCM1.56	*hsp-16.2p* [4-1]

Gateway Expression Constructs

Plasmid	Details	Used to make
pJH23	*unc-17p::sek-1:: tbb-2utr-operon-GFP::H2B::cye-1utr*	*yakEx72*
pJH24	*unc-47p::sek-1:: tbb-2utr-operon-GFP::H2B::cye-1utr*	*yakEx73*
pJH46	*hsp-16.2p::sek-1:: tbb-2utr-operon-GFP::H2B::cye-1utr*	*yakEx121*

CRISPR/Cas9 Constructs to make *sek-1(yak115)*

Plasmid	Details	Used to make
pJH27	Homology repair template with *sek-1* homology arms (from pJH19, pJH20) and *C. briggsae unc-119(+)*	*yak115*
pJH22	sgRNA and Cas9 construct, mutated from pDD162	*yak115*

Appendix 3

Whole Genome Sequence Data Analysis

Whole genome sequencing (WGS) data is often returned as raw data that must be processed to be user-friendly. Researchers can use the tools available at usegalaxy.org to analyze whole genome sequencing data, which is processed on the public Galaxy server (Afgan et al., 2016). This appendix explains the workflows that we modified and utilized to process *C. elegans* WGS data and includes a protocol to process WGS data that the Ailion lab has received. The protocol takes WGS data files (in either FASTQ or BAM format), identifies variants relative to the reference genome, annotates variants, and removes variants that are redundant with other strains (Figure A3.1).

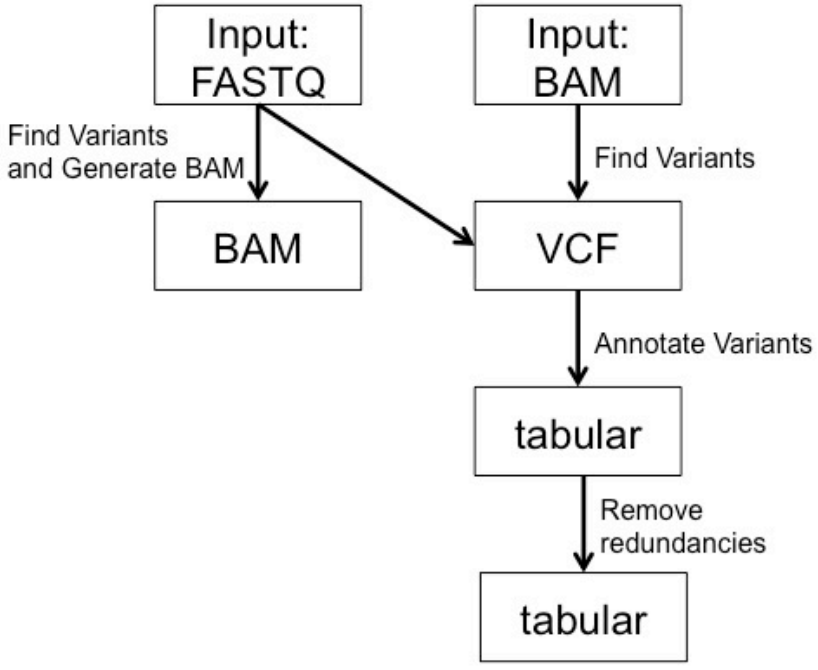

Figure A3.1: Summary of WGS data analysis workflow. File types (boxed) are processed using workflows (arrows and names) to convert WGS data into user-friendly annotated variant files.

Access Galaxy at: usegalaxy.org | Header panel User → Log in

First, it is important to understand the different parts of Galaxy. When you log into Galaxy, there will be a panel on each side of the screen: **Tools**, **History**, and header (panel at the top of the screen). To navigate from different screens of the website back to the homepage, click the Galaxy logo in the upper left-hand side of the screen.

Tools (left hand side of the screen) are pieces of software that Galaxy makes available to use either on its own or within a **Workflow**. Tools are used for functions such as converting file types, manipulating text, or generating statistics. To use a Tool, you must give it appropriate inputs and it will generate outputs. When using a tool, you can use a file in the current **History** (right side of screen). The tool will place the output file in the current History. You can run tools by clicking it, filling out the form (with information such as the input files) and pressing “Execute”. Tools do not delete the input files so it is safe to explore different tools without harming your input data.

The **History** is a list of files that you’re working with. Files are sorted with newest on the top, with 1 being the oldest file found at the bottom of the history. You can rename a History by clicking on it and entering a new one. Files in a History can be viewed by clicking the button that looks like an eyeball, or deleted by clicking the X. To download a file, or take other operations on it, click its name and more options and details will be shown. To switch to another History, click the open book icon at the top of the History panel (or at the top of the page: User → Saved Histories). This will display all of your saved Histories for your account. You can make a history the current History by clicking the “Switch to” button above its name. You are free to switch between

Histories at any time: even if a Tool or Workflow is running against your current History, all of this is happening in the background. No need to worry about accidentally closing the window or reloading the page; it will keep going until it's done.

Workflows are chains of tools: the outputs of one Tool are immediately taken as the input of the subsequent Tool until the final Tool creates the final output.

In the header, click Workflow to see what Workflows are already present in your account. Click on a Workflow to run it. When a Workflow is running, it will generate a file at each point: the files that the Workflow generates between tools are often hidden within the history but the final output is not. If you want to see more about how a Workflow works, you can go to the Workflow page, click on a Workflow, and then click "Edit". This shows a nice graphical representation of how each Tool is chained together to form the Workflow, and also lets you see / modify the parameters given to each tool. Galaxy will ask if you want to save the changes you make, so you can feel free to move things around or change settings and then be sure to tell it to discard changes.

A basic understanding of Tools, History, and Workflow are required to efficiently navigate the Galaxy website to process WGS data with the following protocol.

1. Download the WGS data files onto a computer (such as a lab computer) and make sure these files will get backed up. The WGS file type the lab received from the University of Utah DNA Sequencing Core is BAM.

2a. If the data files you want to upload are smaller than 2GB each, you can upload them directly to Galaxy, detailed below:

Tools → Get Data → Upload File from your computer

A pop-up screen will appear: drag and drop files into screen

At the bottom of the screen:

type (set all): auto-detect (leave as is)

Genome (set all): C. elegans Oct. 2010 WS220 (ce10)

Click Start to upload the files

2b. If the data file is larger than 2GB, it will not upload and you will need to first use a FTP (file transfer protocol) client, such as FileZilla (already on the lab computer). BAM files are typically at least 4GB. To use FileZilla as an FTP client:

Host: usegalaxy.org | username: galaxy username | password: galaxy password

Leave [Port:] empty and press Quick Connect.

In the top part of the screen, it will eventually say Status: Successful

Under [Local Site:] find the folder containing the sequencing data

Highlight the files you want to upload and right click → Upload

In the bottom part of the screen, your files will appear along with progress bars.

Once all the files are successfully transferred (it will take several hours), return to the Galaxy website

Tools → Get Data → Upload File from your computer

In the pop-up screen that appears, click “Choose FTP File”, select all the files and click Start.

In the History panel, the uploaded files will appear with a gray background while they are uploading to the History and the files will have a green background when they are uploaded, which will take only a few minutes.

3. Edit the uploaded files. Click the pencil icon next to a file name within the History. Cut and paste the original file name into the notes and rename the file with the strain name. Also in the notes section you can add the strain genome. Check that the Database/Build is set to WS220 *C. elegans* Oct. 2010 and if not change it so it is set correctly. The WS220 *C. elegans* Oct. 2010 is the most recent release available on Galaxy as of January 2017.

4. The History that these files uploaded to will be “Unnamed History”, so rename it by clicking on it and typing in a more informative name (such as the sequencing method, year, strains sequenced, etc.) and pressing return to save the name. Click on the open book icon in the History panel to view all the Histories. In order to process the input files using the “Find Variant” workflows, these workflows will need a reference genome to align to. Find the History named “WS220.64 reference genome”, which only contains a FASTA file of WS220 *C. elegans* Oct. 2010 named “WS220.64 ch.fa”.

5. It is easiest to keep track of files during the “Find variants” part of the WGS data analysis when each strain is contained in its own History. Create a new History and add the reference genome by dragging and dropping it into the new history. Also add an uploaded sequencing file to the same History and rename the History with the strain

name. Repeat this process until each sequencing file has its own History that also contains the WS220 reference genome file.

6. Sequencing files can be in two formats: FASTQ or BAM. These files must be processed to find variants relative to the reference genome. The first step of finding variants will result in VCF (variant call format) files. If you received FASTQ files, use the "Find Variants (FASTQ to VCF) and Generate BAM" workflow and if you received BAM file, use the "Find Variants (BAM to VCF)" Both workflows use the Unified Genotyper tool to identify variants (DePristo et al., 2011).

The Ailion lab has received WGS data for 14 strains to date: four from Illumina sequencing (2013) and ten from Ion Torrent sequencing (six in 2014, four in 2015). The file type received from Illumina sequencing is FASTQ whereas the Ion Torrent sequencing returned BAM files. For FASTQ files, find variants using Step 6A. If BAM files are received, skip to Step 6B.

6A. **FASTQ files** are FASTA sequence files that contain quality information from sequencing. In order to generate BAM and VCF files, we modified the "CloudMap Unmapped Mutant workflow" (Minevich et al., 2012). We modified this workflow in the following ways: adding a FASTQ groomer at the beginning of the workflow (a tool that reformats the FASTQ input so that it will be recognized as an input in the following tool), deleting all the tools after the variants are found (as they are unnecessary in this protocol), and removing the tools the create statistics files. Additionally, this published

workflow required the input file of a list of candidate genes and we removed this requirement. The resulting modified workflow is named “Find Variants (FASTQ to VCF) and Generate BAM”.

In the workflow menu, select “Find Variants (FASTQ to VCF) and Generate BAM” and click “run”. The two inputs are the FASTA reference genome and the FASTQ sequencing file. Click “Run Workflow”. This will take several hours to run. There will be two files that appear in the History: “Alignment File (BAM)” and “Unified Genotyper on data [#] and data [#] (VCF)”. Rename these files “[strain name] BAM” and “[strain name] VCF”, respectively. You can visualize the BAM file by following the steps in 6B. Skip to Step 7 to annotate the variants.

6B. **BAM (Binary Alignment/Map) files** can be used to visualize the sequencing reads and how they align to the genome. To visualize the BAM file, click on a file in your current History that is format: bam. Click on “display at UCSC main”. On the UCSC Genome Browser page under Custom Tracks, select “pack” and click “refresh”.

The “Find variants (BAM to VCF)” workflow will process BAM files to make VCF files. Find a History that contains an uploaded sequencing file and the reference genome and click “switch to” to make it the Current History. Go to Workflow, find the “Find variants: BAM to VCF”, and click “run”. The workflow will have automatically filled in with the BAM file and the reference genome. Click “Run workflow”. This workflow takes several hours to run. If you received FASTQ files, you already have VCF files and do not need to process the BAM file.

7. Now the variants in the VCF file need to be annotated. The VCF file that will need to be annotated will be “[strain name] VCF”. In the Workflow page, there are several different “Annotate variants” workflows that all contain the base tool SnpEff. SnpEff uses the reference genome set within SnpEff to annotate variants in the VCF input file (Cingolani et al., 2012). The current version of SnpEff (4.0) found in the Tools panel does not contain WS220 and will not work for *C. elegans* WGS data annotation, as one cannot add WS220 to the genome list within this tool. However, all the “Annotate variants” workflows contain an older version of SnpEff where WS220 is accessible to the tool. All of these workflows only take a minute or two to run. The way these workflows differ are:

- If heterozygous annotations are annotated
- If variants in non-protein coding genes are annotated
- How many bases upstream/downstream of a gene that variants are annotated

Running “Annotate variants: base” will return a tabular file that Microsoft Excel can open to view the annotations. Opening this file in Excel will contain annotated variants that are homozygous, heterozygous, and affect a variety of genes: protein coding, pseudogenes, and different RNAs. It will also show variants up to 5000 bases upstream and downstream of genes. To change any of these parameters, click Edit on the workflow and click on the SnpEff tool (box). In the Details panel on the right side of the screen, you can change the annotation of heterozygous versus homozygous variants in the “Filter homozygous / heterozygous changes” section. You can select how many bases upstream or downstream of a gene to annotate in the drop-down menu in the “Upstream / Downstream length” section. If you do modify this (or any other)

workflow, click the gear icon and “Save As” another workflow. If you edit a tool within a workflow, it only affects that tool within that workflow (and not the same tool in other workflows)

Running “Annotate variants: base and “protein_coding only” filter” will return only the variants that affect protein coding genes. This workflow uses a Filter tool after SNPeff. The default parameters result in a file annotating variants up to 5000bp upstream/downstream and annotating both heterozygous and homozygous variants.

The two other “Annotate variants” workflows have the “protein_coding only” filter and only annotate homozygous mutations. These workflows differ by how far away from genes variants are annotated (0 bp or 1000 bp) and their names reflect this parameter. Our recommendation is to run the “Annotate Variants: homozygous, no upstream/downstream” workflow. If the data at the end of this protocol yields no annotations that are promising candidates, use another (less restrictive) “Annotate Variants” workflow. Choose the workflow and hit Run. Then, in the Input Dataset step, select “[strain name] VCF” and click “Run workflow.” The output file will be named “SnpEff on data #” so rename the file with the strain name and some information about the workflow parameters used (for example, put “protein coding changes only (0bp)” in the name if you used the “Annotate Variants: homozygous, no upstream/downstream” workflow).

8. The annotated variants files are tabular files that can be downloaded and viewed using Excel.

Remember, these files contain all the variants relative to the *C. elegans* reference genome. Strains within labs are likely more similar to each other than the reference genome, so annotated variants of different strains can be used to filter out variants that are common between the strains (Figure A3.2).

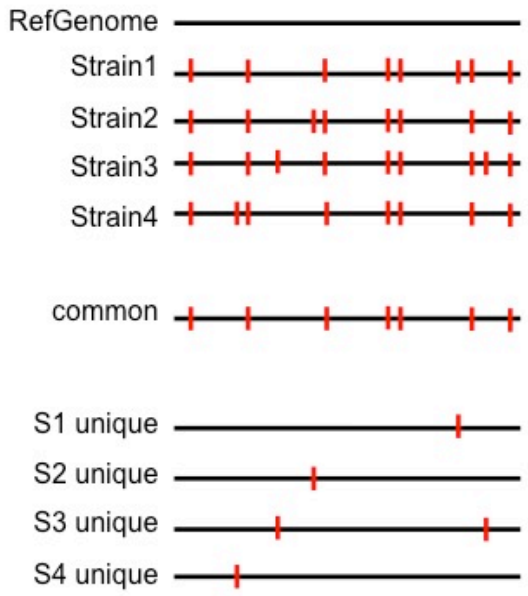

Figure A3.2: Annotated variants can be redundant between strains. Variants (red lines) in genomes (black lines) are called relative to the reference genome. The “Remove redundancies” workflows identify common variants and output files that contain unique variants.

For example, strains XZ1233, XZ1382, XZ1307, and XZ65 from our lab were analyzed using the “Find Variants” and “Annotate Variants: homozygous, no upstream/downstream” workflow. In XZ1233, there are 1192 protein coding changes, which include 122 non-synonymous mutations. By using a workflow to remove variants found in the other strains (called common or redundant variants), we find that only 235 protein coding changes are unique to the XZ1233 strain relative to XZ1382, XZ1307, and XZ65 (Table A3.1). It is possible to filter out common mutations by only using one file to find redundancies but the more files used identifies more common mutations. You can use the “[strain name]: only protein coding changes (0bp)” files in the “0bp up/downstream remove redundancies” History to remove redundancies.

Table A3.1: Removing redundancies reveals unique and common mutations

File	XZ1233 (start file)	XZ1233-XZ1382	XZ1233-XZ1382-XZ1307	XZ1233-XZ1382-XZ1307- XZ65
Protein coding variants	1192	369	264	235
protein coding variants compared to start file	n/a	31% unique 62% common	22.1% unique 77.9% common	19.7% unique 80.3% common
Non-synonymous variants	122	31	25	24
non-synonymous variants compared to start file	n/a	25.4% unique 74.6% common	20.5% unique 79.5% common	19.7% unique 80.3% common

View all the Histories and create a new History. Rename the History “Remove Redundancies”. Add the annotated variant files (format: tabular) by dragging them from their History into the new “Remove Redundancies” History. Make sure the annotated variant files were created with the same “Annotate variants” workflow. In the workflow menu, select a “Remove redundancies (# noise files)” workflow. In each workflow, Input 1 will be the file where you are trying to find unique variants by removing redundancies found in the Input 2 file (or more). Input 1 can be thought of as the “signal” file and the other Inputs as “noise” files. Each “Remove redundancies (# noise files)” workflow is named with how many files you can input as noise files. These workflows take a couple minutes and when they are done, rename the output file, which will be a tabular file.

9. Download and open tabular files using Excel. You can sort the annotated variants by selecting the first row and clicking Data→Filter.

10. There is a finite amount of space available on each user's Galaxy account. Once the sequencing files have been processed using the "Find Variants" workflows, the original files can be deleted from the account. The original files are very large and deleting them will free up a lot of space.

If you don't want to delete the files from Galaxy, I recommend opening a second account and moving the original uploaded sequencing files there and then deleting these files from the lab account. While these original files were theoretically backed up during Step 1, it takes many hours to upload the original files and it doesn't hurt to have this data stored redundantly. To do this, view all the Histories and switch to the History with the original uploaded files. Return to the main page and clear the gear icon within the History panel. Click the "Share or Publish" option. In the page that appears, select "Share with a user" and enter the email address of the second account. Log out of the lab account and into the second account. In the History panel, click the gear icon and select "Histories Shared with Me". Click on the name of the shared History and click view. Under the Header panel, click "Import History". This will copy the History and switch it to the Current History immediately. Return to the original lab account and find the History that you shared with the second account.

If you want to delete the entire History, make it the current History, click the gear icon, and select "delete." A box will appear to ask if you are sure and click yes to permanently delete it. If you want to delete files within a History, click the X to the right of the file name. To permanently delete this file, navigate to the History name and click

deleted to view the deleted files. Within the yellow box above the deleted file name, click “permanently delete.”

This protocol details *C. elegans* WGS data starting from BAM files and results in tabular files where annotated variants are reported. The variants found by the Unified Genotyper tool (within our “Find Variants” workflow) and other variant calling tools available on Galaxy cannot identify copy number variants. Copy number variants are segments of DNA 1kb or larger and can be deletions, duplications, or insertions. The XZ1233 strain was sequenced because it contained the *yak42* mutation that suppressed *egl-30(gf)*. The files from this WGS data analysis protocol did not identify the *yak42* variant. *yak42* is a 3.9 kb deletion of *sek-1* and was identified by scrolling through the BAM file on the UCSC Genome Brower. These workflows were able to identify the variants such as *ksr-1(ox314)*, *ksr-1(yak10)*, and *grk-2(yak18)*.

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