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Identifying Human 21st Chromosome Orthologs Required for Neuronal Function in *Caenorhabditis elegans*

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Identifying Human 21st Chromosome Orthologs Required for Neuronal Function in *Caenorhabditis elegans*

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Identifying Human 21st Chromosome Orthologs Required for Neuronal Function in *Caenorhabditis elegans*

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Down syndrome, caused by trisomy of the 21st chromosome, leads to lifelong cognitive impairment, characterized by alterations to neural cells and circuits, brain tissue, and behavior. Efforts to understand this disorder first require an understanding of the genes encoded on the 21st chromosome. The small roundworm, *Caenorhabditis elegans*, is an excellent model to uncover the *in vivo* function of human 21st chromosome orthologs.

The first chapter of my dissertation provides background on Down syndrome—how it is thought to arise, the phenotypes it causes within the nervous system, which genes and pathways may underlie these phenotypes, and a brief survey of the established mouse models used to interrogate the disorder. I conclude by arguing that the more tractable model organism, *C. elegans*, can be used to complement research with rodents and that worm is a particularly fruitful system for initial gene characterization.

In my next chapter, I discuss two systematic screens I performed on human 21st chromosome orthologs in worm: the first, a behavioral screen to identify genes

broadly affecting nervous system function; the second, a pharmacological screen to identify genes involved in synaptic signaling. In these two screens, I identified a handful of candidate genes, several of which had not previously been linked to the nervous system in any animal.

In Chapter 3, I discuss one of these novel candidate genes, *mtq-2*, that emerged from my screen. I provide a fuller characterization of it and show that it is required for normal nervous system function, normal excitatory neurotransmission, and that it functions, specifically, in cholinergic neurons to mediate its effects. I also discuss the potential genetic pathways in which *mtq-2* may work to control synaptic vesicle release in excitatory motor neurons. Here, I present evidence that suggests *mtq-2* may function upstream of or in conjunction with at least one Gα signaling protein, Gα_O. I conclude by sharing preliminary results on overexpression of *mtq-2* and suggest future directions for inquiry.

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CHAPTER ONE

INTRODUCTION

OVERVIEW

In 1866 a young medical doctor, John Langdon Down, published a brief in which he characterized patients with a consistently similar set of traits: cognitive impairment, facial dysmorphology, and speech and motor deficits amenable to improvement by "systematic training" (Down, 1866). This early system of phenotypic classification denotes the start of the field of Down syndrome research, though it would take nearly another hundred years and seismic advances in biotechnology before a collection of phenotypes could be linked to the presence of an extra chromosome.

Trisomy of the 21st chromosome is the most common type of autosomal aneuploidy with long-term viability (Antonarakis, Lyle, Dermitzakis, Reymond, & Deutsch, 2004; Gardiner et al., 2010). Children born with Down syndrome present with an array of phenotypes ranging from the superficial—epicanthic folds, flat nasal bridges, Brushfield spots—to those that severely affect quality of life—intellectual disability, low muscle tone, septal heart defects, and early onset Alzheimer disease (Korenberg et al., 1994). Down was a thorough, careful observer—the phenotypes he enumerated in the 19th century are still those most widely associated with the syndrome bearing his name; yet unraveling how complete (or in rarer cases, partial) triplication of the 21st chromosome results in Down syndrome remains elusive.

Of the phenotypes associated with Down syndrome, most have variable penetrance with two notable exceptions: cognitive impairment and neonatal hypotonia. These phenotypes are inevitable outcomes for patients afflicted with the disorder (Korenberg et al., 1994). Hypotonia, or low muscle tone, is

characterized by laxity of the ligaments, which hinders normal motor development. Though children with Down syndrome are ultimately able to reach major motor milestones, they do so at a delayed rate and by relying on compensatory strategies to move (Agiovlasitis, McCubbin, Yun, Pavol, & Widrick, 2009; Carr, 1970; Melyn & White, 1973; Rigoldi, Galli, Mainardi, Crivellini, & Albertini, 2011). Movement is thus, more difficult and more energetically taxing for the individual with Down syndrome (Agiovlasitis et al., 2009).

The second fully penetrant phenotype, cognitive impairment, causes more severe difficulties for Down syndrome patients, their families, and society. Most individuals with Down syndrome are moderately to severely intellectually disabled, with a median IQ of ~50 (Chapman & Hesketh, 2000; Dierssen, Herault, & Estivill, 2009). Though useful as a diagnostic metric, an IQ score cannot—and does not—capture the complexity of human behavior. As I will discuss in a later section, the cognitive profile of individuals with Down syndrome is distinct from other types of intellectual disability and correlates to deficits in specific brain regions.

Of final note, nearly all individuals with Down syndrome develop the characteristic pathophysiological hallmarks of Alzheimer's disease in adulthood. Understanding how neurodegeneration arises and progresses in older patients with Down syndrome may provide insight into more global mechanisms of Alzheimer's disease in the general population.

What follows is a survey of Down syndrome—the most common hypotheses underlying its cause, its effects on specific areas of the nervous

system, and how invertebrate models—*C. elegans* in particular—can augment our understanding of Down syndrome related genes and genetic pathways. Though trisomy of the 21st chromosome can affect every major organ system, a discussion of the non-neurological phenotypes among people with Down syndrome is beyond my scope.

CAUSES OF DOWN SYNDROME

Historically, two hypotheses were advanced to explain the development of Down syndrome due to the presence of a third 21st chromosome—the amplified developmental instability hypothesis and the "gene dosage" hypothesis (Dierssen, 2012; Pritchard & Kola, 1999). More recent ways of thinking—with which I agree—argue for a more nuanced interpretation. I will discuss both historical and contemporary arguments here.

The amplified developmental instability hypothesis posits that the phenotypes associated with Down syndrome are the result not of overexpression of any specific gene, but rather a generalized disruption of homeostasis triggered by an excess of genetic material (Shapiro, 1975). Central to the argument is that organisms develop within the context of tight gene dosage parameters. Further, the most variable anthropometric traits are also those that are most susceptible to environmental influences. Human height, for instance, is a highly variable trait that is highly susceptible to environmental effects. An extra chromosome disturbs this evolutionarily optimized homeostasis causing the individual to become more susceptible to environmental stress. Traits that are already vulnerable to environmental stress (e.g. variable traits like height) are those most

likely to become perturbed in Down syndrome. This argument largely sprung from the observation that different aneuploidies converge on similar traits. While this certainly has some element of truth to it—Williams syndrome (a small deletion of the 7th chromosome) and Down syndrome both result in heart defects and cognitive impairment—there are critical distinctions. Individuals with Down syndrome exhibit discrepancies in their working memory profiles, generally performing poorly on verbal tasks, less so on visuospatial tasks (Dierssen, 2012). However, this pattern is of relative visuospatial dominance is reversed in individuals with Williams syndrome, which suggests that alterations to specific genes or genomic regions underlie specific patterns of memory impairment (Conners, Moore, Loveall, & Merrill, 2011; Vicari, Bellucci, & Carlesimo, 2005).

The development of early onset Alzheimer's disease is also unique to Down syndrome, but not to other aneuploidies. When Shapiro advanced the amplified developmental instability hypothesis in the 1970s, the average life expectancy for people with Down syndrome was about 30-35 years old (Deaton, 1973; Thase, 1982). Individuals with Down syndrome do not display the pathological markers of Alzheimer's—Aβ plaques and neurofibrillary tangles—until their 40s and do not present with dementia until their 50s, thus obscuring the inevitability of developing Alzheimer's disease to researchers of past generations (Hartley et al., 2015).

The idea of dose sensitive genes encoded on the 21st chromosome has often been framed in contrast to the developmental instability hypothesis—an unfortunate false dilemma (Pritchard & Kola, 1999). Though the human 21st chromosome is the smallest chromosome, it has an average, overall gene density

(Hattori et al., 2000). Genes encoding long non-coding RNA (lnc-RNA) are enriched relative to other chromosomes, while genes encoding microRNA (miRNA) are depleted; the density of pseudogenes and protein-coding genes is average (Letourneau & Antonarakis, 2012). Conservative estimates state the number of protein coding genes on HSA21 as around 220 (Gupta, Dhanasekaran, & Gardiner, 2016; Letourneau & Antonarakis, 2012; Nordquist, Smith, & Pierce, 2018).

Determining the function of these genes and understanding how they might contribute to Down syndrome is a non-trivial task. It is, therefore, understandable that Down syndrome researchers would be motivated to identify a minimal susceptibility region or "critical region" for the disorder. In 1989, researchers identified two rare individuals with Down syndrome who were only partially trisomic for the 21st chromosome. By mapping their 21st chromosomes, Rahmani identified a small region shared by both patients, which was dubbed—perhaps prematurely—the Down syndrome critical region (DSCR) (Rahmani et al., 1989). Twenty years later, the Korenberg lab found over 30 individuals with Down syndrome whose condition sprung, again, from rare partial trisomies of the 21st chromosome. Using fine resolution mapping techniques, they discovered that there was no single region of conservation among all of the Down syndrome patients (Korbel et al., 2009). In other words, no one chromosomal region along the 21st chromosome was sufficient to explain all—or even most—of the phenotypes associated with Down syndrome (Korbel et al., 2009). Importantly, the Korenberg group identified multiple regions that correlated to intellectual

disability in Down syndrome, which argues against the involvement of a single critical region causative for cognitive impairment.

Studies using mouse models to investigate the Down syndrome critical region have also been conflicted. In mouse, genes orthologous to those on the human 21st chromosome are distributed along the 16th, 17th, and 10th chromosomes. The purported Down syndrome critical region lies within the mouse 16th chromosome. In 2004, the Reeves group created two very useful mouse models to investigate the function of the critical region: Ms1Rhr/Ts65Dn and Ts1Rhr (**Figure 1.1**) (Olson, Richtsmeier, Leszl, & Reeves, 2004). In Ms1Rhr/Ts65Dn, a mouse segmentally monosomic for the DSCR (Ms1Rhr) was crossed to Ts65Dn, a previously established mouse model of Down syndrome. Selected Ms1Rhr/Ts65Dn progeny were, thus, disomic for genes within the critical region. In contrast, the Ts1Rhr mouse was trisomic *only* for the DSCR. Olson and Reeves examined craniofacial skeletal abnormalities—a hallmark of human Down syndrome and the mouse models of it—and found that Ms1Rhr/Ts65Dn mice had craniofacial dysmorphology similar to Ts65Dn mice, arguing against the necessity of the DSCR. Ts1Rhr mice, which were trisomic only for the DSCR, more closely resembled their euploid littermates, arguing against the sufficiency of the DSCR. The results of this study indicate that overexpression of genes outside of the DSCR contribute to facial phenotypes in Down syndrome and that overexpression of the DSCR alone is not sufficient to cause these facial phenotypes.

In a subsequent study, Olson and Reeves examined hippocampal behaviors and cell physiology of both the Ts1Rhr and Ms1Rhr/Ts65Dn mice

(Olson et al., 2007). They found no differences between euploid and Ts1Rhr mice on performance of the Morris water maze, a measure of hippocampal function; nor did they find any differences in evoked hippocampal long-term potentiation, a measure of synaptic plasticity. However, "subtraction" of the DSCR region in the Ms1Rhr/Ts65Dn mice led to behavioral improvements on hippocampal-dependent tasks. Collectively, these results argue for the necessity of some genes or genomic elements in the DSCR region for hippocampal development and function, but not its sufficiency.

Belichenko and Mobley also used Ts1Rhr mice to examine the sufficiency of the DSCR to cause both cellular and behavioral deficits (N. P. Belichenko et al., 2009). The Mobley group found that Ts1Rhr mice had altered hippocampal synaptic plasticity and deficits on several other hippocampal-dependent behaviors. Notably, the Mobley group and the Reeves examined different behaviors and recorded cell responses from different regions of the hippocampus, which explains—in part—their differing conclusions.

Strictly speaking, for the idea of a single DSCR to be true, patients lacking this region should not display a majority of Down syndrome phenotypes. This assertion is unsupported (Korbel et al., 2009; Lyle et al., 2009). Of course genes within this region contribute to the cognitive phenotypes in Down syndrome, but there are other regions of susceptibility in both humans (Korbel et al., 2009; Lyle et al., 2009) and mouse models (Zhang et al., 2014). I am inclined to agree with the sentiments of Gupta and Gardiner that the continued use of the term "Down syndrome critical region" is misleading and should be avoided (Gupta et al., 2016).

How best, then, to consider Down syndrome? Antonarakis proposed a sensible model (Antonarakis et al., 2004), which acknowledges the following points: 1) there exist dose-sensitive genes or non-genic sequences on the 21st chromosome; 2) the ability of these dose-sensitive genes or regions to contribute to Down syndrome phenotypes may either be allele-specific on non-specific; 3) dose-sensitive elements on the 21st chromosome may interact with, and dysregulate, non-21st chromosome genes.

MOLECULAR MECHANISMS OF DOWN SYNDROME

Cognitive impairment in Down syndrome is rooted in alterations to nervous system tissues, cells, and circuits. Though the cause of Down syndrome is complex, several genes and pathways have been linked to specific neurological phenotypes in Down syndrome. Here I discuss several key studies that provide possible molecular mechanisms for the emergence of specific Down syndrome phenotypes. Understanding how a supernumerary chromosome causes a disorder as varied and complex as Down syndrome is—admittedly—a daunting, but worthwhile endeavor. By considering the neurological phenotypes of Down syndrome as discrete parts, we can learn about specific genes and pathways—their contribution to the disease state—and armed with this knowledge, propose rational therapies.

Neurodevelopment

Differences between a euploid and Down syndrome brain become evident during embryonic neurodevelopment. Specifically, Down syndrome affected

brains are reduced in overall size; this has been observed as early as the fourth month of gestation (Engidawork & Lubec, 2003; Guihard-Costa, Khung, Delbecque, Ménez, & Delezoide, 2006). The reduction in overall brain size correlates to neuronal hypocellularity, most likely due to failures of neurogenesis *in utero* (Schmidt-Sidor, Wisniewski, Shepard, & Sersen, 1990).

Interestingly, not all regions of the brain are equally affected by Down syndrome: at birth, the cerebellum is disproportionately small; in adulthood, the hippocampus (Dierssen, 2012). Mouse models of Down syndrome recapitulate these phenotypes. In one of the Down syndrome mouse models, Ts65Dn, there are fewer hippocampal granule neurons during early development and fewer proliferating hippocampal cells postnatally (Clark, Schwalbe, Stasko, Yarowsky, & Costa, 2006; Insausti et al., 1998; Lorenzi & Reeves, 2006; Rueda, Mostany, Pazos, Flórez, & Martínez-Cué, 2005). Contestabile found in both human fetal brains and mouse that proliferating hippocampal cells spent a reduced amount of time in the synthesis (S) phase of the cell cycle, suggestive of a general timing deficit in the cell cycle (Contestabile et al., 2007).

The conspicuously small cerebellum in Down syndrome also correlates to hypocellularity, specifically, a decreased number of cerebellar granule cells and Purkinje cells (Baxter, Moran, Richtsmeier, Troncoso, & Reeves, 2000). Offering at least part of an explanation, Roper found that cerebellar granule cells have a weakened, dose-dependent response to the mitogenic effects of Sonic hedgehog (SHH) (Roper et al., 2006). Using a Down syndrome mouse model, Das discovered that boosting the SHH signal in newborn mice with a synthetic activator, SAG 1.1, corrected adult cerebellar hypocellularity (Das et al., 2013).

The restoration of near normal cell counts in the adult cerebellum, unexpectedly, did not correspond to any improvements in cerebellar dependent behavioral tasks, but rather, led to stark improvements in hippocampal-dependent cognitive performance (Das et al., 2013). It is not clear why Down syndrome mice have an attenuated response to SHH, nor why improving cerebellar morphology improved hippocampal-dependent behavioral tasks, but this is a rich area for future investigation.

Altered Neurotransmitter Systems

Abnormalities in embryonic neurodevelopment underlie much of the brain dysmorphology observed in infants with Down syndrome, and strongly contribute to cognitive impairment in patients with Down syndrome. Yet the brain is a remarkably responsive organ, subject to constant, fine change throughout a lifespan. While prenatally "curing" Down syndrome is neither ethically, nor practically possible, identifying postnatal treatments for Down syndrome—cognitive deficits in particular—is a reasonable goal. Many of the current therapeutic options for enhancing cognition in Down syndrome rely on correcting altered neurotransmitter systems—GABAergic and serotonergic, in particular.

Down syndrome mice have fewer hippocampal excitatory synapses and altered connectivity of hippocampal inhibitory synapses (Dierssen, 2012). These morphological differences likely underlie the increased hippocampal inhibition and alterations in hippocampal synaptic plasticity observed in rodents. Given these results, several labs have sensibly attempted to pharmacologically curtail

excessive GABA inhibition with $GABA_A$ receptor antagonists or selective negative allosteric regulators. In mice, reducing excessive GABA signaling leads to impressive recovery of hippocampal physiological and behavioral deficits (Braudeau et al., 2011; Fernandez et al., 2007). Regrettably, these improvements in cognition following pharmacological inhibition of excessive GABA signaling have not extended to humans with Down syndrome; and clinical trials have been shelved for the moment (Antonarakis, 2017).

In addition to alterations in GABAergic signaling, Down syndrome humans and mice also show alterations in serotonergic signaling, at least partially caused by reduced expression of the serotonergic receptor, 5-HT1A (Bar-Peled et al., 1991; Bianchi et al., 2010; Risser, Lubec, Cairns, & Herrera-Marschitz, 1997). Increasing extracellular serotonin with selective serotonin reuptake inhibitors (SSRIs) is linked to hippocampal neurogenesis (Malberg, Eisch, Nestler, & Duman, 2000; Santarelli et al., 2003). Several groups working with Down syndrome mouse models have found that treatment with SSRIs restores hippocampal cell proliferation deficits and 5-HT1A receptor expression and improves learning and memory behaviors (Bianchi et al., 2010; Clark et al., 2006). Given that SSRIs are safe, widely used, and already approved, they are an attractive therapeutic option and likely to be explored in clinical trials in the near future.

Neurodegeneration

Nearly all individuals with Down syndrome develop Aβ plaques, neurofibrillary tangles, and neurodegeneration—in short, all of the

neuropathological hallmarks of Alzheimer's disease—by their 40s; by age 55-60, most exhibit symptoms of dementia (Hartley et al., 2015). Though the molecular underpinnings of Alzheimer's disease are complex, amyloid precursor protein, APP, encoded on the 21st chromosome, is strongly associated with the disorder. Proteolysis of APP generates several fragments, the most pathologic of which is Aβ. The amyloid hypothesis (longstanding, though not without controversy) posits that accumulation of Aβ initiates the development of Alzheimer's disease (Querfurth & LaFerla, 2010). Overexpression of APP In Down syndrome is thought to lead to increased generation of the toxic Aβ product (Prasher et al., 1998). The link between Alzheimer's disease, or "premature senility," and Down syndrome is old and (J. Fraser & Mitchell, 1876). The importance of APP to both disorders has also been appreciated for decades (Glenner & Wong, 1984; Masters et al., 1985). Nevertheless, there are some differences between Down syndrome-associated Alzheimer's disease (DSAD) and the more common late-onset Alzheimer's disease (LOAD) found in the euploid population. For one, Aβ deposition in patients with Down syndrome begins much earlier than in the general population, with some studies finding Aβ deposits in Down syndrome brains as early as the age of 12 (Lemere et al., 1996). Further, less neurodegeneration is observed in DSAD than in LOAD, though patients with Down syndrome have fewer neurons to begin with (Mann, Yates, Marcyniuk, & Ravindra, 1987).

Overexpression of other 21st chromosome genes may also contribute to Alzheimer's-associated neurodegeneration observed in older adults with Down syndrome. For instance, the kinase, DYRK1A, phosphorylates several splicing

factors, which predispose the tau protein to abnormal hyperphosphorylation (F. Liu et al., 2008). A microtubule associated protein, tau normally promotes the assembly and stability of microtubules—the beams of the cell. Hyperphosphorylated tau, however, loses its affinity for microtubules and is more prone to forming the aggregates observed in neurofibrillary tangles (Querfurth & LaFerla, 2010). Liu discovered that DYRK1a phosphorylated tau at several sites *in vitro*; these sites were also hyperphosphorylated in the brains of adults with Down syndrome (F. Liu et al., 2008).

Finally, expression levels of synaptojanin, SYNJ1, have been linked to Alzheimer's disease. SYNJ1 is a phosphatase, which acts on a key synaptic molecule, PIP_2 (phosphatidylinositol 4,5-biphosphate) (Cremona et al., 1999). Interestingly, decreased levels of SYNJ1 in haplosufficient mice appear to protect against the synaptotoxicity of Aβ (Berman et al., 2008).

An increased understanding of 21st chromosome genes—or related pathways—that mediate neurological development, neuronal function, or neurodegeneration, thus, enriches our understanding of Down syndrome and can potentially lead to therapeutic options to address aspects of the disorder.

APPROACHES TO IDENTIFYING CANDIDATE GENES AND PATHWAYS

There are several different approaches to identifying candidate genes or pathways of interest to Down syndrome. Results from any one of these strategies can lead to fertile lines of inquiry in others.

HSA21 Gene Expression

Traditionally, it was assumed that an additional 21st chromosome would result in the expression of triplicated genes at 1.5 times the typical amount. As transcriptome profiling has demonstrated, this is largely, but not entirely true (Lockstone et al., 2007; Mao, Zielke, Zielke, & Pevsner, 2003). More recently, the Herwig group found that trisomy of the 21st chromosome was associated with dysregulation of genes on other chromosomes (Vilardell et al., 2011). Overexpression of transcription factors or other proteins exerting broad regulatory roles could possibly explain some of this genome wide dysregulation. Several transcription factors are known to be encoded on HSA21, including three—*RUNX1*, *BACH1*, and *ERG*—associated with an increased risk for leukemia in Down syndrome (Dierssen, 2012).

Functional Classification

Functional classification of genes on the 21st chromosome can also be used to predict candidate genes contributing to neurological phenotypes in Down syndrome. For instance, several ion channel subunits are encoded on the 21st chromosome, including a subunit of the G-protein coupled inwardly rectifying potassium channel, *GIRK2*. Cooper found that mice trisomic for *Kcnj6*, the gene encoding *GIRK2*, displayed deficits in hippocampal-dependent learning and memory tasks and had altered synaptic plasticity—phenotypes also observed in Down syndrome mouse models (Cooper et al., 2012). Genes encoding cell adhesion molecules have also been proposed as candidates contributing to neurological phenotypes in Down syndrome; indeed, overexpression of the Down syndrome cell adhesion molecule, *DSCAM*, has been shown to inhibit

dendritic branching in mouse hippocampal neurons (Alves-Sampaio, Troca-Marín, & Montesinos, 2010). Reduced dendritic branching is another characteristic of adults with Down syndrome (Becker, Mito, Takashima, & Onodera, 1991). Finally, genes encoding proteins with master regulatory functions can contribute to neurodevelopmental and cognitive deficits in Down syndrome. The kinase, *DYRK1A*, is one of the best characterized genes in this category; I will discuss its functional role in the following section.

Genetic Dissection

Genetic dissection is a tried and true approach for discovering candidate genes contributing to a phenotype of interest. In the baldest of terms, this technique involves breaking a gene and observing the effects. Given their relative simplicity, invertebrates have been particularly well-suited to this approach; several genes critical for neurodevelopment and cognitive function in Down syndrome were initially characterized in invertebrates.

Genetic screening in *Drosophila* identified the gene *mnb* (*minibrain*), required for postembryonic neurogenesis (Tejedor et al., 1995). Subsequent research in mice, identified the ortholog of *mnb*, *Dyrk1a,* and found that it, too, played a pivotal role in neurogenesis (Shindoh et al., 1996; W. J. Song, Chung, & Kurnit, 1997). *DYRK1A*—dual-specificity tyrosine phosphorylation-regulated kinase 1A—is involved in neurodevelopment, motor function, and cognition and has been rigorously studied within the context of Down syndrome (Ahn et al., 2006; Altafaj et al., 2001).

Genetic screening in *C. elegans* has also been fruitful. In his seminal work, Sydney Brenner identified and characterized *unc-26*, later shown to encode synaptojanin (Brenner, 1974). A phosphatase, synaptojanin plays a key role in synaptic vesicle recycling and modulates levels of second messenger molecules within the synapse. In mice, overexpression of SYNJ1 causes memory deficits reminiscent of those in Down syndrome mouse models (Voronov et al., 2008).

C. ELEGANS AS A MODEL ANIMAL FOR NEUROGENETICS

Superficially, *C.elegans* is an unassuming animal. In the wild, this small, transparent roundworm lives out its brief life amid decomposing organic matter. Yet its simplicity belies its utility for understanding the genetic basis of nervous systems. Adult hermaphroditic worms have 302 neurons, stereotypically located. Systematic ablation of these neurons has revealed the function of many of them. Displaying truly heroic effort, White determined the wiring diagram of *C.elegans* by reconstructing serial electron micrographs (J. G. White, Southgate, Thomson, & Brenner, 1986). Thus, we know the identity of every neuron, its location within the animal, and with whom it communicates.

Because of the compactness of the worm's nervous system, deficits in a specific behavior can often indicate deficits in a specific neuron or circuit. The specificity of behavioral outputs in worm is impressive; and combined with the power of modern genomics and genetics, remarkable. Examination of simple behaviors of mutant worms has helped to identify several genes with critical roles in the nervous system, including the first GABA transporter (*unc-47*) and

the first glutamate transporter, (*eat-4*) (Rankin, 2002). Importantly, both of these genes were later found to be conserved in mammals.

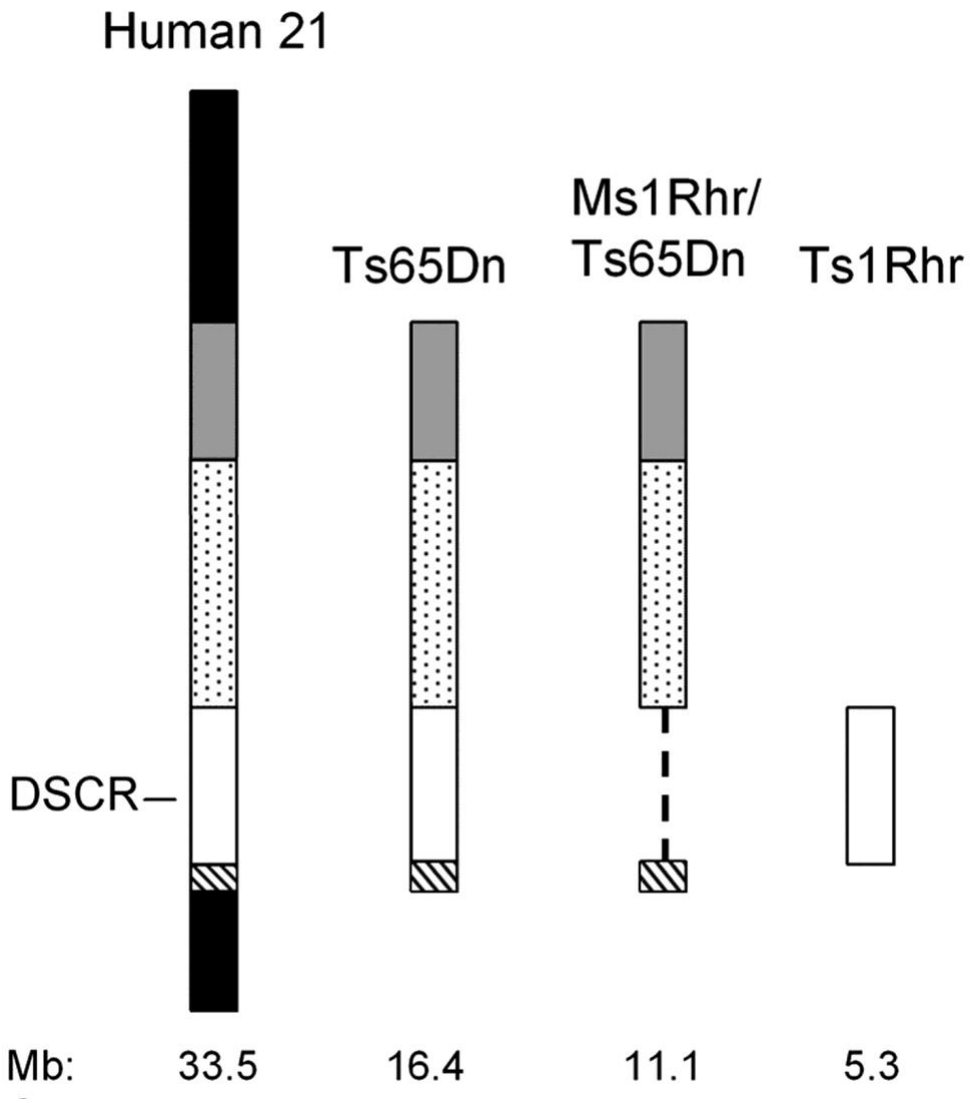

Figure 1.1. Mouse models of Down syndrome and the Down syndrome "critical region." Depicted here is the human 21st chromosome (Human 21) with the purported Down syndrome critical region (DSCR) shown in white. The corresponding region triplicated in Ts65Dn, a popular mouse model of Down syndrome, is adjacent. In Ms1Rhr/Ts65Dn, the DSCR has been crossed out. In Ts1Rhr, only the DSCR is triplicated. From (Olson et al., 2004).

CHAPTER TWO

SYSTEMATIC FUNCTIONAL CHARACTERIZATION OF 21ST CHROMOSOME ORTHOLOGS IN *CAENORHABDITIS ELEGANS*[1]

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ABSTRACT

Individuals with Down syndrome have neurological and muscle impairments due to an additional copy of the human 21st chromosome (HSA21). Only a few of the ~200 HSA21 genes encoding protein have been linked to specific Down syndrome phenotypes, while the remainder are understudied. To identify poorly characterized HSA21 genes required for nervous system function, we studied behavioral phenotypes caused by loss-of-function mutations in conserved HSA21 orthologs in the nematode *Caenorhabditis elegans*. We identified ten HSA21 orthologs that are required for neuromuscular behaviors: *cle-1* (*COL18A1*), *cysl-2* (*CBS*), *dnsn-1* (*DONSON*), *eva-1* (*EVA1C*), *mtq-2* (*N6ATM1*), *ncam-1* (*NCAM2*), *pad-2* (*POFUT2*), *pdxk-1* (*PDXK*), *rnt-1* (*RUNX1*), and *unc-26* (*SYNJ1*). We also found that three of these genes are required for normal release of the neurotransmitter acetylcholine. This includes a known synaptic gene *unc-26* (*SYNJ1*), as well as uncharacterized genes *pdxk-1* (*PDXK*) and *mtq-2* (*N6AMT1*). As the first systematic functional analysis of HSA21 orthologs, this study may serve as a platform to understand genes that underlie phenotypes associated with Down syndrome.

INTRODUCTION

Down syndrome (DS) is the most common genetic cause of intellectual disability, occurring with an incidence as high as 1 in ~700 live births in the United States (Canfield et al., 2006). Although DS is defined by a variety of symptoms, all individuals with DS display varying degrees of intellectual disability, including learning and memory problems, and upon middle age they develop Alzheimer's-like dementia (Coyle, Oster-Granite, Reeves, & Gearhart, 1988). In addition, all individuals with DS experience neuromuscular symptoms. For instance, DS is the leading cause of neonatal heart defects, leading to a high infant mortality rate without surgical intervention (Korenberg et al., 1994; Vis et al., 2009). Another common symptom of DS, hypotonia (muscle weakness), causes deficits in both gross and fine motor skills, leading people with DS to have trouble speaking, writing, and moving efficiently (Pitetti, Climstein, Mays, & Barrett, 1992).

For over fifty years, researchers have known that an extra copy of the 21st chromosome (Human somatic autosome 21, HSA21) underlies DS (Jacobs, Baikie, Court Brown, & Strong, 1959). However, the precise mechanisms by which trisomy 21 causes DS-associated phenotypes are largely unknown. An early hypothesis explaining the link between DS and trisomy 21 argued that the burden of the extra genetic material strains cellular processes in the DS patient, resulting in all DS symptoms (Patterson, 2009; Shapiro, 1975). Yet research on rare individuals with partial trisomy 21 has shown that the amount of extraneous genetic material does not readily account for the collection of symptoms associated with DS nor their degree of manifestation (Korenberg et al., 1994; Lyle

et al., 2009). A second hypothesis posited that a region containing about 30 protein-coding genes on HSA21, termed the Down Syndrome Critical Region (DSCR), is responsible for all DS-associated phenotypes. This was based on the genomic area of overlap shared between two individuals with partial trisomy 21 who still displayed many DS phenotypes (Rahmani et al., 1989). A mouse model (Ts1Rhr) containing trisomy of only DSCR orthologous genes was developed to investigate the role of the DSCR (Olson et al., 2004). Many, but not all of the phenotypes expected for DS were observed in this mouse model, suggesting that the DSCR is important, but not solely responsible for the whole set of DS-associated phenotypes (N. P. Belichenko et al., 2009; Olson et al., 2004). Mouse models of DS trisomic for syntenic regions of HSA21 other than the DSCR have also been instrumental in the analysis of the genetic origins of DS-related phenotypes, including craniofacial defects (Richtsmeier, Baxter, & Reeves, 2000; Richtsmeier, Zumwalt, Carlson, Epstein, & Reeves, 2002), cerebellar cell loss (Baxter et al., 2000), as well as synaptic and hippocampal circuit dysfunction (P. V. Belichenko et al., 2004; Demas, Nelson, Krueger, & Yarowsky, 1998; Escorihuela et al., 1995; Holtzman et al., 1996; Reeves et al., 1995).

Studies focusing on individual orthologous genes on HSA21 in animal models have also provided insight into the genetic basis of DS phenotypes. Single gene studies are attractive because the underlying genetic contributions of a phenotype are more readily dissected; single genes can also offer clearer insight into pharmacotherapeutic targeting of specific gene products (Dierssen, 2012). Determining the biological function of a single gene can be approached with either a loss-of-function or gain-of-function (e.g. overexpression) approach. Both

have merit. For genes for which there is no or incomplete redundancy, loss-of-function experiments provide valuable insight into gene function. Several HSA21 orthologous genes were initially characterized in this way in invertebrate models. Early work conducted in *Drosophila melanogaster*, for instance, revealed that the gene *mnb* (*minibrain*) played a critical role in postembryonic neurogenesis (Tejedor et al., 1995). Subsequent research linked fly *mnb* to its mammalian orthologue, *DYRK1A*; and, as in fly, loss of *Dyrk1a* in mice was also shown to result in abnormal neurogenesis (Shindoh et al., 1996; W. J. Song et al., 1997; 1996). Intriguingly, overexpression of *Dyrk1a* alone in a mouse transgenic model results in neurodevelopmental delay, motor abnormalities, and spatial memory defects, which suggest a potential role in DS-associated cognitive impairment (Ahn et al., 2006; Altafaj et al., 2001). Similarly, the neuronal role of the basic helix-loop-helix protein, *SIM2* (single-minded family bHLH transcription factor 2) on HSA21 was also initially identified in fly. Mutations in the fly ortholog, *sim*, impair development of cells in the midline of the central nervous system (Crews, Thomas, & Goodman, 1988; Thomas, Crews, & Goodman, 1988). Subsequent experiments identified the mouse homolog and found that a targeted deletion of the gene led to craniofacial malformations (H. Chen et al., 1995; Shamblott, Bugg, Lawler, & Gearhart, 2002). Overexpression of *Sim2* in mouse also recapitulated behavioral aspects of established mouse models of DS including reduced exploratory behaviors and hypersensitivity to pain (Chrast et al., 2000). Single gene studies and invertebrate models, thus, complement research with traditional DS mouse models that overexpress combinations of many HSA21 genes.

Despite this progress, the *in vivo* function of a majority of genes on HSA21 remains unknown. It is impractical to perform a systematic analysis of gene function through gene knock down in mouse models. Therefore, we set out to gain knowledge about roles of HSA21 genes by studying the function of HSA21 orthologs in the nematode *Caenorhabditis elegans*, a more tractable genetic model.

C. elegans is a well-established genetic model and exhibits sequence similarity with at least 42% of genes associated with human disease (Culetto & Sattelle, 2000). Additionally, the *C. elegans* nervous system is compact, with only 302 neurons (White et al., 1985). Because any one neuron may be connected to any other by only 6 or fewer synapses, defects in even a single neuron are often detectable with behavioral phenotypes such as locomotion (B. L. Chen, Hall, & Chklovskii, 2006). Proper function of the muscular feeding organ, called a pharynx, also requires precise communication between neurons and muscle. Defects in pharyngeal pumping have helped identify a conserved vesicular glutamate transporter (e.g. *eat-4*) and nicotinic cholinergic receptor subunits (Avery & You, 2012). Finally, *C. elegans* synapses are also similar to those of mammals. Genetic screens using the acetylcholinesterase inhibitor aldicarb in worm have identified genes critical for synaptic function in mammals (Richmond, 2005).

Because the *in vivo* functions of HSA21 genes have not been systematically studied, we suspected that some HSA21 genes with important neuronal or muscle roles were overlooked. The goal for our study was to determine HSA21 orthologs involved in neuronal and/or muscle function. To this end, we analyzed behavioral phenotypes induced by loss-of-function mutations and found that

disruption of several uncharacterized HSA21 orthologs in worm yielded significant neuromuscular phenotypes. To determine if any HSA21 orthologs were involved in neurotransmission, we performed a second screen on loss-of-function mutants measuring their sensitivity to the acetylcholinesterase inhibitor aldicarb. We identified three genes involved in neurotransmitter release: *unc-26 (SYNJ1)*, which has previously been characterized, and *mtq-2 (N6AMT1)* and *pdxk-1 (PDXK)*, both of which have received minimal attention. The results from our behavioral and pharmacological screens may inform predictions for which genes contribute to the neuronal and muscle phenotypes associated with DS.

RESULTS

Estimate of HSA21 Protein-Coding Genes

To determine a conservative number of protein-coding genes on the human 21st chromosome, we queried both Ensembl and Human Gene Nomenclature Committee (HGNC) databases and selected only those proteins reviewed by SwissProt. This public database provides manual curation and review for each protein, which ensures high quality, non-redundant entries (UniProt Consortium, 2015). This strategy yielded a total number of 213 protein-coding genes on the 21st chromosome **APPENDIX A, APPENDIX D, APPENDIX E**.

Determining HSA21 orthologs in C. elegans

To identify putative worm orthologs of protein-coding genes on the human 21st chromosome (HSA21), we relied on OrthoList, a publicly available

database. OrthoList compiles results from a meta-analysis of four orthology prediction programs—Ensembl Compara, OrthoMCL, InParanoid, and Homologene (Shaye & Greenwald, 2011). OrthoList predicts at least one worm ortholog for 85 of the 213 HSA21 protein-coding genes (*blue* and *light-grey wedges* in **Figure 2.1**). HSA21 encodes 48 predicted keratin proteins, which have no orthologs in worm (Shaye & Greenwald, 2011). Excluding these keratin-encoding genes, *C. elegans* has at least one predicted orthologous gene to 52% of the remaining HSA21 genes (**Figure 2.1)**. To focus our screen on genes that most likely share conserved functions, we chose to study orthologous genes existing in any relationship except many-to-many as defined by the InParanoid algorithm (Sonnhammer & Östlund, 2015). This included one-to-one, one-to-many, and many-to-one relationships. By this measure, InParanoid identifies 47 unique HSA21 genes with predicted orthologs in worm (three wedges highlighted by *red arc* in **Figure 2.1**). These 47 HSA21 human genes are represented by 51 genes in worm.

The imperfect evolutionary matchup of HSA21 human genes with *C. elegans* genes results in many human genes being orthologous to multiple worm genes (**Figure 2.1** and see *Orthologs* sheet in **APPENDIX A**. For instance, the human gene *CBS* is predicted to be orthologous to four paralogs: *cysl-1*, *cysl-2*, *cysl-3*, and *cysl-4*. There was only one case of paralogous human genes in our analysis: both *RRP1* and *RRP1B* human genes are predicted to be orthologous to the single worm gene *C47E12.7*. Of the 47 HSA21 human genes, 19 are orthologous one-to-one with worm genes, 25 are orthologous many-to-one with 25 worm genes, and 3 are orthologous one-to-many with 8 worm genes.

An additional 38 HSA21 human genes had one or more orthologs predicted by the Ortholist algorithm in *C. elegans* (depicted by *light-grey wedge* in **Figure 2.1** and listed in **APPENDIX D.** Because most of these HSA21 human genes are predicted to be orthologous to multiple worm genes, orthology of the 38 human genes expands to represent 111 worm genes in total. For example, the human gene *ABCG1* is predicted to be orthologous to four paralogs: *wht-2*, *wht-3*, *wht-6*, and *wht-8*. In four cases, distinct but paralogous human genes are orthologous to the same worm gene. For instance, both *OLIG1* and *OLIG2* human genes are both predicted to be orthologous to *hlh-16* in worm. To focus our screen, we did not pursue these genes in this study.

Lastly, we found that an additional 128 protein-coding genes on HSA21 have no predicted Ortholist otholog in *C. elegans* (depicted by *black wedge* in **Figure 2.1** and listed in **APPENDIX E**.

Taken together, our analysis above shows that discounting the keratin genes, about half of the 213 HSA21 human genes are predicted to be orthologous to genes in *C. elegans*. We focused on the set of 47 HSA21 human genes represented by 51 InParanoid orthologs in worm in this study.

HSA21 orthologs required for viability in *C. elegans*

Before embarking on our behavioral screen using mutants, we first sought to determine which of the 51 HSA21 predicted orthologs are required for viability using RNAi (**APPENDIX B**). Previous large-scale and small-scale RNAi screens studied 38 of the 51 genes (Ceron et al., 2007; Cui et al., 2008; A. G. Fraser et al., 2000; Gottschalk et al., 2005; Kamath et al., 2003; Maeda, Kohara,

Yamamoto, & Sugimoto, 2001; Simmer et al., 2003; Sönnichsen et al., 2005). These studies reported that RNAi treatments caused viability phenotypes for 15 of these orthologs that severely effect development, growth, and/or reproduction. These include embryonic and larval lethal, larval arrest, severe growth defects, and sterility phenotypes. For an additional 24 orthologs, these studies reported that RNAi treatment caused an apparent wild-type phenotype. Out of the 51 HSA21 worm orthologs, 14 were not studied in previous RNAi screens, in part, because no RNAi reagent was available at the time.

To confirm and extend these results we performed RNAi on all 51 HSA21 orthologs for which an RNAi clone was currently available using 45 unique RNAi treatments (**APPENDIX B**). We found concordance for 82% or 37 out of the 45 genes tested. Only 4% or two of our RNAi treatments produced results discrepant from previous studies. RNAi treatment of *irk-1* (*KCNJ6*) or *chaf-2* (*CHAF1B*) were previously reported to cause severe viability phenotypes whereas we found no effect for both treatments (Gottschalk et al., 2005; Kamath et al., 2003; Nakano, Stillman, & Horvitz, 2011; Sönnichsen et al., 2005). We found that 14 out of 16 of the genes that caused inviability in our study or in previous studies when knocked down, including *irk-1* and *chaf-2*, lacked a publically available viable loss-of-function mutant. This finding is consistent with the idea that these genes are probably essential. Two additional genes, *dip-2* and *dnsn-1*, had corresponding viable predicted null mutants, but we and others found that they caused inviability when knocked down by RNAi (Simmer et al., 2003; Kamath et al., 2003); perhaps this reflects a viability phenotype with low penetrance. We acknowledge that additional genes may later prove to represent

essential genes, especially if the gene lacks a corresponding null mutant, e.g. *pdxk-1*. Overall our RNAi screen confirms most viability phenotype results from previous studies and suggests that at least 33% or 15 out of 45 of tested HSA21 predicted orthologs are inviable when reduced in function in *C. elegans.*

HSA21 orthologs required for neuromuscular behaviors

Having identified which HSA21 orthologs are probably essential, we turned next to investigate the *in vivo* function of nonessential HSA21 orthologs. We tested every publically available, viable mutant, which represented 27 genes in total (**APPENDIX C**). We performed a phenotypic screen, selecting a battery of three behavioral assays to probe neuromuscular function. Overall, we identified ten mutants that were significantly defective in performance in these assays**.** To better link genotype to phenotype, we generated transgenic rescue lines for any mutant deficient in any of the three behaviors and/or tested additional mutant alleles if available.

We first examined short-term locomotion with a radial dispersion assay, which provides a general metric of locomotor function over 10 minutes. In this assay, the distance displaced over time reflects a combination of locomotor traits including frequency of body bends, reversal rate, and coordination (Topalidou et al., 2017). Aside from the well-characterized uncoordinated mutant *unc-26*, an ortholog of synaptojanin (Harris, Hartwieg, Horvitz, & Jorgensen, 2000), we also identified two other mutants with rescuable deficits in radial dispersion: *cysl-2 (CBS)* and *rnt-1 (RUNX1)* (**Figure 2.2**).

Second, we examined the longer-term behavior of mutants with an exploration assay. This assay measures not only the general locomotor ability of worms, but also their tendency to explore a cultivation plate seeded with bacterial food over the course of 16 hours. While foraging, worms alternate between two states: roaming—an active state characterized by a burst of locomotion associated with a search for food—and dwelling—a more passive state associated with feeding or resting after satiation. Time spent in roaming and dwelling states depends on integrating internal neuromodulatory cues with external sensory cues. The absence of such sensory transduction leads to extended dwelling as observed in the *tax-4* mutant, which encodes a cyclic nucleotide-gated channel subunit (Fujiwara, Sengupta, & McIntire, 2002; Greene et al., 2016). In contrast, mutants with constitutive sensory input, such as the *egl-4* mutant, show extended roaming (Fujiwara et al., 2002). Extended dwelling or roaming compared to wild-type behavior can be quantified by counting the number of squares that a worm traverses over 16 hours (**Figure 2.3**).

As expected, we found that the uncoordinated mutant *unc-26* (*SYNJ*) moved poorly in the exploration assay. We also identified the following four mutants with rescuable defects in extended dwelling: *eva-1 (EVA1C), dnsn-1 (DONSON), mtq-2 (N6AMT1),* and *pdxk-1 (PDXK1)* (**Figure 2.3**). These mutants appeared to move superficially similar to wild type, suggesting sensory-motor integration deficits. We rescued the exploration behavior of each mutant by transformation with wild-type copies of corresponding transgenes (**Figure 2.3**).

To obtain a measure of neuromuscular activity that was distinct from locomotion, we performed a feeding assay. *C. elegans* pumps bacterial food into

its gut through a feeding organ called a pharynx that uses muscles and neurons distinct from those needed for locomotion. The rhythmic contractions and relaxations of the pharynx require precise coordination between pharyngeal neurons and muscle. We identified the following four genes required for normal pharyngeal pumping: *cle-1 (COL18A1), mtq-2 (N6AMT1), ncam-1 (NCAM2),* and *pad-2 (POFUT2)* (**Figure 2.4**). Independent null alleles of *mtq-2* displayed similar deficits in pharyngeal pumping (**Figure 2.4**). Transformation with wild-type copies of each gene rescued the slow pumping phenotype of each mutant (**Figure 2.4**).

In summary, by screening three behaviors, we identified ten genes total with rescuable defects in one or more behaviors designed to probe neuromuscular function. Several among these genes—*dnsn-1 (DONSON), pdxk-1 (PDXK),* and *mtq-2 (N6AMT1)*—are poorly characterized, yet may mediate nervous system function, development, or both.

HSA21 orthologs required for proper synaptic function

To determine the potential role of HSA21 predicted orthologs in neurotransmission, we tested whether the same set of loss-of-function mutants displayed altered sensitivity to paralysis by the acetylcholinesterase inhibitor aldicarb. Many genes critical for key steps in synaptic function have been identified by testing mutants for altered sensitivity to aldicarb (Richmond, 2005). Aldicarb causes paralysis of worms by chronically activating body-wall muscle after the prolonged presence of acetylcholine at the neuromuscular junction. Mutants defective in any aspect of synaptic transmission show altered sensitivity

to paralysis by aldicarb. For instance, mutants defective in acetylcholine release show resistance to paralysis by aldicarb due to decreased levels of acetylcholine in the cleft (Mahoney, Luo, & Nonet, 2006). Conversely, mutations that increase acetylcholine release lead to increased sensitivity to paralysis (Gracheva et al., 2006; McEwen, Madison, Dybbs, & Kaplan, 2006).

We identified three mutants that displayed significant resistance to paralysis by aldicarb: *unc-26 (SYNJ1)*, *mtq-2* (*N6AMT1*), and *pdxk-1* (*PDXK*). Compared to wild type, these three mutant strains took longer to paralyze when treated with aldicarb (**Figure 2.5**). By contrast, 24 other mutants became paralyzed at a similar rate as wild-type worms. Although we measured the 180-minute time course of paralysis for all 27 mutants, we plot only the percent moving on aldicarb at the 180-minute time point for ease of comparison (**Figure 2.5**). Transformation of *mtq-2* and *pdxk-1* mutants with wild-type copies of these genes rescued aldicarb sensitivity for each mutant (**Figure 2.6**). As a complementary strategy to the mutant screen, we also tested aldicarb sensitivity for HSA21 predicted orthologs using RNA interference. With this independent approach, we confirmed the aldicarb resistance of *pdxk-1 (PDXK-1)* and observed suggestive results for *mtq-2 (MTQ-2)* (**Figure 2.7**).

We found that RNAi treatment targeting *ncam-1* provided resistance to aldicarb; however, we also found that a null allele of *ncam-1* failed to show any resistance to paralysis by aldicarb (**Figure 2.5** and **Figure 2.7**) suggesting that our RNAi result for *ncam-1* was a false positive. This result is consistent with a previous RNAi study that found negative results for *ncam-1* on aldicarb (Sieburth et al., 2005).

Resistance to aldicarb is primarily caused by presynaptic deficiencies in acetylcholine release or postsynaptic deficiencies in receptor response (Mahoney et al., 2006). To distinguish between these possibilities, we measured the sensitivity of mutants to paralysis by levamisole, a potent agonist for the nicotinic cholinergic receptor (Lewis, Wu, Berg, & Levine, 1980). Levamisole causes paralysis of wild-type worms by chronically activating body-wall muscle irrespective of synaptic release defects. Mutants resistant to paralysis by levamisole are most commonly defective in cholinergic receptor signaling or muscle function.

We found that *mtq-2*, *unc-26,* and *pdxk-1* mutants all displayed wild-type-like or hypersensitive responses to levamisole (**Figure 2.6**). These results suggest that resistance to aldicarb among these three mutants is due to defective presynaptic release of neurotransmitter.

Expression pattern of HSA21 orthologs

We identified ten genes linked to rescuable neuromuscular defects in our study (**Table 2.1**). Five genes were previously shown to be expressed in the nervous system: *cle-1* (*COL18A1*), *eva-1* (*EVA1C*), *ncam-1* (*NCAM2*), *pad-2* (*POFUT2*), *rnt-1* (RUNX1) and *unc-26* (*SYNJ*) (Ackley et al., 2001; Fujisawa, Wrana, & Culotti, 2007; Hunt-Newbury et al., 2007; McKay et al., 2003; Menzel et al., 2004; Schwarz, Pan, Voltmer-Irsch, & Hutter, 2009). An additional four genes had no reported expression patterns: *cysl-2 (CBS)*, *dnsn-1 (DONSON), mtq-2 (N6AMT1),* and *pdxk-1 (PDXK)*. To identify the tissues in which these genes may function, we generated mCherry transcriptional reporter strains. We found that

mtq-2 expressed exclusively throughout the nervous system. Expression overlapped with a subset of cholinergic neurons in the head and throughout the ventral nerve cord as defined by the *Punc-17::GFP* reporter (**Figure 2.8**). Expression of *mtq-2* mCherry reporter was not observed in GABAergic neurons defined by the *Punc-47:GFP* reporter (**Figure 2.8**). Interestingly, our mCherry reporter for *pdxk-1* expressed in GABAergic, but not cholinergic neurons (**Figure 2.8**). Our transcriptional reporter for *dnsn-1* expressed robustly within the developing embryo, but not within the adult nervous system (**Figure 2.8**). This suggests a potential developmental role for this gene. Lastly, we found that our mCherry *cysl-2* reporter expressed broadly throughout head and body-wall muscle (**Figure 2.8**).

DISCUSSION

Our research predicts new functional annotation of genes on the human 21st chromosome by characterizing *in vivo* roles of putative orthologs in *C. elegans*. Within the field of Down syndrome, research has primarily focused on the functions of contiguous groups of genes using mice, such as those included in the so-called Down syndrome critical region (DSCR) (X. Jiang et al., 2015). Meanwhile, the vast majority of HSA21 genes remain functionally uncharacterized. One proven approach to reveal the function of large numbers of genes, even at the level of the whole genome, is to study their roles in more tractable invertebrate models such as *C. elegans*. Although several large-scale reverse genetics screens in *C. elegans* have begun to uncover novel *in vivo* roles for thousands of genes, they have focused less explicitly on neuronal phenotypes

and excluded more genes than may be generally acknowledged (A. G. Fraser et al., 2000; Kamath et al., 2003; Sieburth et al., 2005; Vashlishan et al., 2008). For instance, two seminal whole-genome RNAi screens tested only 33 of the 51 HSA21 orthologs focused on in the current study (A. G. Fraser et al., 2000; Kamath et al., 2003). These studies primarily focused on viability phenotypes such as gross development and fertility. We expanded this analysis to test an additional eight orthologs not tested in these seminal or subsequent RNAi studies (Ceron et al., 2007; Gottschalk et al., 2005; Nakano et al., 2011; Pujol et al., 2001; Rual et al., 2004; Sönnichsen et al., 2005). With the addition of our current study, out of the 51 HSA21 orthologs, only five genes remain unstudied using RNAi or mutant in any study due to lack of reagents: *D1086.9* (*CLIC6*), *H39E23.3* (*CLIC6*), *Y54E10A.11* (*LTN1*), *Y74C10AL.2* (*TMEM50B*), and *ubc-14* (*UBE2G2*). Overall, however, our results were consistent with earlier studies. We found only two discrepancies: previous studies found that RNAi targeting *irk-2* and *chaf-2* caused sterility while we found no effect (Gottschalk et al., 2005; Kamath et al., 2003).

Other seminal RNAi screens in *C. elegans* searched for genes critical for synaptic transmission. Even though these screens were prodigious, testing 2,068 genes in total, only five of the HSA21 orthologs: *mbk-1*, *itsn-1*, *ncam-1*, *K02C4.3*, and *wdr-4* (Sieburth et al., 2005; Vashlishan et al., 2008) were examined. These studies reported negative results for all five genes in agreement with our RNAi and mutant findings here. Notably, the synaptic RNAi screens did not test the three genes that we found to be critical for normal synaptic transmission: *mtq-2*, *pdxk-1*, and *unc-26*. Thus, despite the whole-genome and large-scale nature of

previous screens, many genes with important *in vivo* functions likely remain to be discovered using *C. elegans*. Because our study largely replicated results from previous studies, we are optimistic that a systematic approach with *C. elegans*, as used here, may reveal the function of important sets of genes involved in human polygenic or chromosomal disorders.

We also performed several behavioral screens to identify HSA21 predicted orthologs that function in nervous system and muscle in ways that previous reverse-genetic screens may have missed. This included a radial dispersion assay that reflects short-term locomotor ability, an exploration assay that reflects longer-term sensory and motor integration, and a feeding assay that probes an independent neuromuscular circuit. We identified 10 mutants with deficiencies in these behaviors that we were able to rescue and/or confirm with independent alleles (**Table 2.1**): *cle-1* (*COL18A1*), *cysl-2* (*CBS*), *dnsn-1* (*DONSON*), *eva-1* (*EVA1C*), *mtq-2* (*N6AMT1*), *ncam-1* (*NCAM2*), *pad-2* (*POFUT2*), *pdxk-1* (*PDXK*), *rnt-1* (*RUNX1*), and *unc-26* (*SYNJ*). These 10 orthologs are alluring candidate genes that may mediate nervous system development and/or function and should, thus, be prioritized for further study in worm and mammalian model systems.

In general, our targeted screen of HSA21 orthologs revealed three primary findings for the 10 HSA21 orthologs. First, we found genes with established links to nervous system function (e.g. *cle-1*, *eva-1*, *ncam-1*, and *unc-26*). For instance, *cle-1* (*COL18A1*), *eva-1* (*EVA1C*), and *ncam-1* (*NCAM2*) and have all been linked to deficits in axon guidance in worm. The type XVIII collagen gene, *cle-1*, mediates both neuronal and axonal migrations (Ackley et al., 2001). *eva-1* encodes a Slit

receptor required for axon guidance, and *ncam-1* encodes a neural cell adhesion molecule that functions redundantly with other cell adhesion molecules to direct axonal outgrowth (Fujisawa et al., 2007; Schwarz et al., 2009). Additionally, the *unc-26* (*SYNJ*) mutant showed resistance to aldicarb but sensitivity to levamisole indicative of presynaptic defects. This is consistent with its known role in synaptic vesicle recycling (Harris et al., 2000).

Second, we uncovered novel neuronal functions for the otherwise well-characterized HSA21 ortholog *rnt-1* (*RUNX1*). In mammals, the runt-related transcription factor *RUNX1* plays a vital role in development, including regulation of the developing nervous system (Wang & Stifani, 2017). In worm, the runt-related transcription factor *rnt-1* has also been studied for its role in general development, including cell proliferation and male tail development (Hajduskova, Jindra, Herman, & Asahina, 2009; Nimmo, Antebi, & Woollard, 2005). Interestingly, loss of *rnt-1* may also cause defects to phasmid neuron morphology; however, this was not linked to a detectable behavioral phenotype (Hajduskova et al., 2009). The locomotor deficiency that the *rnt-1* mutant displayed in our radial dispersion assay might be due to developmental defects affecting the nervous system.

Third, whereas the majority of Down syndrome research has focused on well-studied genes, we also uncovered novel neuronal functions of three poorly characterized HSA21 orthologs that have weak or nonexistent links to the nervous system: *dnsn-1* (*DONSON*), *mtq-2* (*N6AMT1*), and *pdxk-1* (*PDXK*). These three genes are so uncharacterized that they only had placeholder names when we started our study. To encourage their study, we renamed them based on their

predicted orthologs: *C24H12.5* to *dnsn-1*, *C33C12.9* to *mtq-2*, and *F57C9.1* to *pdxk-1*. Below, we highlight their functions in the limited context of their orthologs in other species.

DONSON was only recently found to encode a novel fork protection factor that underlies microcephalic dwarfism, yet it remains deeply understudied (Reynolds et al., 2017). The worm ortholog, *dnsn-1*, is equally uncharacterized. We found *dnsn-1* mutants to exhibit extended dwelling in an exploration assay and observed expression of *dnsn-1* in the developing embryo, but not the adult animal. Thus, our data are suggestive of a role within the nervous system and possibly within the developing nervous system.

PDXK phosphorylates vitamin B6, converting it to PLP (pyridoxal-5'-phosphate), a key cofactor in the metabolism of hundreds of enzymatic reactions, including synthesis of neurotransmitters (Cao, Gong, Tang, Leung, & Jiang, 2006; Shetty & Gaitonde, 1980). Loss of *PDXK* in mice causes pre-weaning lethality, so its behavioral role has not yet been studied in mammals (Mouse Genome Informatics and the International Mouse Phenotyping Consortium (IMPC)). Interestingly, *PDXK* is predicted to be haploinsufficient in mammals and is a candidate dose-sensitive gene contributing to phenotypes in Down syndrome (Antonarakis, 2016). We found that *pdxk-1* mutant worms showed general deficiencies in behaviors as well as specific defects in synaptic transmission as determined by aldicarb screening. Our finding that the *pdxk-1* mutant was readily paralyzed by levamisole suggests a presynaptic role for *pdxk-1* in the nervous system.

To our knowledge, MTQ-2 has not been implicated in nervous system function in any animal model. The mammalian ortholog of *mtq-2*, *N6AMT1,* was originally named on the basis of the presence of an amino acid motif (D/N/S)PP(Y/FW) which is characteristic of adenine methyltransferases (Bujnicki & Radlinska, 1999; Kusevic, Kudithipudi, & Jeltsch, 2016). However, no evidence of adenine methylation by MTQ-2 protein has been found in either yeast or mouse, suggesting that *N6AMT1* may be a misnomer (P. Liu et al., 2010; Ratel et al., 2006). Instead, MTQ-2 has been shown to post-translationally modify ERF1 (eukaryotic release factor 1) by methylating a universally conserved glutamine residue on ERF1 (Heurgué-Hamard et al., 2006); (Polevoda, Span, & Sherman, 2006). More recently, the mouse ortholog of MTQ-2 was shown to methylate many additional substrates *in vitro* and CHD5 (chromodomain helicase DNA-binding protein 5) and NUT (nuclear protein in testis) *in vivo*. This raises the possibility that MTQ-2 may modulate more proteins than previously thought (Kusevic et al., 2016). Here we have identified behavioral phenotypes of *mtq-2* mutants that support a neuronal role in *C. elegans*. Additionally, we found that a reporter for *mtq-2* widely expresses throughout the nervous system, specifically in a subset of cholinergic neurons. The high conservation (47% amino acid identity between human isoform 1 and worm) of MTQ-2 as well as many of its predicted interacting partners warrant more detailed study to determine how it functions in synaptic transmission. *C. elegans* may provide an especially informative system to study *mtq-2* as deletion of *N6AMT1* causes embryonic lethality in KO mice (P. Liu et al., 2010).

There is a rich body of literature in the field of Down syndrome devoted to exploring the underlying genetic contributions of the disorder. Single gene studies have helped to elucidate the role of individual genes such as *DYRK1A*, both as a transgenic single gene triplication and within a trisomic context, and have led to the development of pharmacotherapeutic treatments (Ahn et al., 2006; Altafaj et al., 2001; Shindoh et al., 1996; W. J. Song et al., 1996). Mouse models have also been instrumental in parsing discrete, chromosomal regions and uncovering genetic pathways that may be amenable to therapeutic targeting (P. V. Belichenko et al., 2015; Das et al., 2013; X. Jiang et al., 2015; Olson et al., 2004; 2007). More recently, bioinformatic approaches have been used to explore the transcriptional landscape of DS and highlight candidate genes that may contribute to DS phenotypes (Letourneau et al., 2014; Sturgeon, Le, Ahmed, & Gardiner, 2012). Our results provide initial clues on the function of several uncharacterized HSA21 orthologs that add to established findings. Conclusions on the precise mechanism of action of these genes and their possible relevance to DS will benefit from more detailed study in *C. elegans*—including examination of possible phenotypes induced by overexpression—as well as further investigation using traditional mouse models and human cell line approaches.

MATERIALS AND METHODS

Strains

C. elegans were grown on NGM (nematode growth media) agar plates seeded with OP50 bacteria at 20° C as described (Brenner, 1974). N2 Bristol served as wild type and was also used for outcrossing (Brenner, 1974). For a list

of outcrossed strains, refer to **APPENDIX F**. The following strains were used is this study: BB3 adr-*2(gv42)* III; CB211 *lev-1(e211)* IV; CZ18537 *zig-10(tm6127)* II; DR97 *unc-26(e345)* IV; EK228 *mbk-1(pk1389)* X; FX1756 *pad-2(tm1756)* III; FX2021 *rcan-1(tm2021)* III; FX2626 *Y105E8A.1(tm2626)* I; FX2657 *nrd-1(tm2657)* I; FX3322 *dnsn-1(tm3322)* II; FX3565 *mtq-2(tm3565)* II; FX6706 *B0024.15(tm6706)* V; FX776 *sod-1(tm776)* II; GA503 *sod-5(tm1146)* II; MT1072 *egl-4(n477)* IV; NS3026 *ikb-1(nr2027)* I; PR678 *tax-4(p678)* III; PS2627 *dgk-1(sy428)* X; RB1489 *D1037.1(ok1746)* I; RB2097 *set-29(ok2772)* I; RB2436 *cysl-4(ok3359)* V; RB2535 *cysl-2(ok3516)* II; RB870 *igcm-1(ok711)* X; RB899 *cysl-1(ok762)* X; RB979 *dip-2(ok885)* I; VC200 *rnt-1(ok351)* I; VC201 *itsn-1(ok268)* IV; VC3037 *mtq-2(ok3740)* II; VC40866 *pdxk-1(gk855208)* I; VC868 *eva-1(ok1133)* I; VC943 *cle-1(gk421)* I; VH860 *ncam-1(hd49)* X.

Transgenic Strains

We used standard techniques to generate constructs used to make the following transgenic strains:

JPS610	*vxEx610 [mtq-2p::mtq-2::mtq-2 UTR + myo-2p::mCherry::unc-54 UTR]; mtq-2 (tm3565)*
JPS611	*vxEx611 [mtq-2p::mCherry::unc-54 UTR + unc-122p::GFP]*
JPS612	*vxEx611 [mtq-2p::mCherry::unc-54 UTR + unc-122p::GFP]; vsIs48 [unc-17::GFP]*
JPS621	*vxEx621 [mtq-2p::mtq-2::mtq-2 UTR + myo-2p::mCherry::unc-54 UTR]; mtq-2 (tm3565)*
JPS906	*vxEx611 [mtq-2p:mCherry::unc-54 UTR + unc-122p::GFP]; oxIs12 [unc-47p::GFP + lin-15(+)]*
JPS929	*vxEx929 [pdxk-1p::mCherry::unc-54 UTR]*
JPS989	*vxEx929 [pdxk-1p::mCherry::unc-54 UTR]; vsIs48 [unc-17::GFP]*
JPS990	*vxEx929 [pdxk-1p::mCherry::unc-54 UTR]; oxIs12 [unc-47p::GFP + lin-15(+)]*

JPS1044 *vxEx1044 [WRM615bF01 + myo-2p::mCherry::unc-54UTR]; B0024.15(tm6706)*

JPS1045 *vxEx1045 [WRM615bF01 + myo-2p::mCherry::unc-54UTR]; B0024.15(tm6706)*

JPS1046 *vxEx1046 [WRM061aD07 + myo-2p::mCherry::unc-54UTR]; rnt-1 (ok351)*

JPS1047 *vxEx1047 [WRM061aD07 +myo-2p::mCherry::unc-54UTR]; rnt-1 (ok351)*

JPS1048 *vxEx1048 [pdxk-1p::pdxk-1::pdxk-1UTR; myo-2p::mCherry::unc-54 UTR]; pdxk-1 (gk855208)*

JPS1049 *vxEx1049 [pdxk-1p::pdxk-1::pdxk-1UTR; myo-2p::mCherry::unc-54 UTR]; pdxk-1 (gk855208)*

JPS1051 *vxEx1051 [WRM0611dD03 + myo-2p::mCherry::unc-54UTR]; eva-1(ok1133)*

JPS1052 *vxEx1052 [WRM0611dD03 + myo-2p::mCherry::unc-54UTR]; eva-1(ok1133)*

JPS1056 *vxEx1056 [WRM069bF04 + fat-7p::GFP]; cle-1 (gk421)*

JPS1057 *vxEx1057 [WRM069bF04 + fat-7p::GFP]; cle-1 (gk421)*

JPS1058 *vxEx1058 [WRM0640bC10 + myo-2p::mCherry::unc-54UTR]; pad-2 (tm1756)*

JPS1059 *vxEx1059 [WRM0640bC10 + myo-2p::mCherry::unc-54UTR]; pad-2 (tm1756)*

JPS1060 *vxEx1060 [WRM0637bA01 + myo-2p::mCherry::unc-54 UTR]; cysl-2 (ok3516)*

JPS1061 *vxEx1061 [WRM0637bA01 + myo-2p::mCherry::unc-54 UTR]; cysl-2 (ok3516)*

JPS1062 *vxEx1062 [WRM0619dG03 + fat-7p::GFP]; ncam-1 (hd49)*

JPS1063 *vxEx1063 [WRM0619dG03 + fat-7p::GFP]; ncam-1 (hd49)*

JPS1064 *vxEx1064 [cysl-2p::mCherry::unc-54 UTR]*

JPS1065 *vxEx1065 [dnsn-1p::mCherry::unc-54 UTR]*

JPS1066 *vxEx1066 [WRM0634aG08 + myo-2p::mCherry::unc-54 UTR]; dnsn-1 (tm3322)*

JPS1067 *vxEx1066 [WRM0634aG08 + myo-2p::mCherry::unc-54 UTR]; dnsn-1 (tm3322)*

RNA interference

We performed RNAi by feeding as described (Timmons, Court, & Fire, 2001). All RNAi clones were obtained from SourceBioScience (Nottingham, UK). First, RNAi-expressing AMP-resistant bacteria were cultured overnight at 37°C with shaking in LB broth containing ampicillin (50 mg/mL) to prevent contamination of liquid cultures. The following day, ~100 μL of bacterial liquid culture was seeded on NGM plates containing 1-mM isopropyl ß-D-1-thiogalactopyranoside (IPTG) to induce expression of exogenous RNA by the T7 promoter. Once a bacterial lawn had sufficiently grown, a mixed age population of BZ1272 *nre-1(hd20) lin-15b(hd126)* double-mutant worms was placed in a 2:1 mixture of bleach and 1-M NaOH to kill bacteria and post-embryonic worms. This strain was selected due to its heightened sensitivity to RNAi in the nervous system (Schmitz, Kinge, & Hutter, 2007). Eggs were allowed to hatch and grow over the next week at 20^{0}C and were observed for the following week. This period covers two generations. The first generation of RNAi-treated worms experienced post-embryonic effects of the RNAi treatment, while the second generation also experienced maternal and pre-embryonic effects. We scored viability phenotypes including embryonic lethality, larval lethality, and larval arrest and sterility in either the P0 or F1 generation. Control-treated worms were fed L440 background-strain bacteria that harbored an empty RNAi vector.

Behavioral assays

All behavioral assays were performed blind to the genotype or RNAi treatment and conducted at room temperature (~20°C).

Radial Dispersion Assay:

To measure radial distance traveled, we placed 5-8 day-one adult worms in the center of a 10-cm diameter plate thinly seeded with OP50 bacteria. The distance traveled from the center of the plate was measured at 10 minutes (Topalidou et al., 2017). A minimum of 30 worms were tested per genotype on at least two separate days.

Exploration Assay:

A single L4-stage worm was placed on a 3.5-cm diameter plate thinly seeded with OP50 bacteria and allowed to crawl freely. After 16 hours, we removed the worm and used the worm track to count how many of 69 squares the explored across. At least 15 worms per genotype were tested on at least two separate days.

Aldicarb Assay:

Sensitivity to the acetylcholinesterase inhibitor aldicarb was quantified as described (Mahoney et al., 2006). At least 25 day-one adult staged worms were evaluated per trial. For the aldicarb assay using RNAi-treated worms, worms were examined at a single time point (100 minutes) on 1-mM aldicarb and scored for paralysis. Trials were performed blind to RNAi treatment and in triplicate for two generations (if viable as F1). For the aldicarb assay performed on mutant worms, the number of paralyzed worms on 1-mM aldicarb was noted every half hour for three hours. A worm was considered paralyzed when it showed neither spontaneous movement nor movement in response to being prodded three times

on the head and tail with a platinum wire. Assays were repeated a minimum of three times.

Levamisole Assay:

Sensitivity to the acetylcholine receptor agonist levamisole was measured as described (Lewis et al., 1980). At least 25 day-one adult-staged worms were placed on plates treated with 800-μM levamisole. We scored worms for paralysis every ten minutes for one hour. Assays were performed in triplicate.

Pharyngeal Pumping Assay:

We quantified the pumping rate of day-one staged worms by eye for thirty seconds under a stereomicroscope at 100x magnification using a handheld counter. A single pump was defined as the backward movement of the grinder (Albertson & Thomson, 1976; Raizen, Lee, & Avery, 1995). At least 30 worms per genotype were analyzed.

Statistical Analysis:

For radial dispersion, exploration, and pharyngeal pumping assays, we compared values to the wild-type control with a one-way ANOVA with a Bonferroni correction to evaluate select comparisons or Dunnett's test when all comparisons were to wild type. Aldicarb and levamisole responses of mutants were compared to wild-type control with a two-way ANOVA and a Bonferroni correction for select comparisons or Dunnet's test when all comparisons were to wild type. We used GraphPad Prism7 software. To minimize false positives from

our screen, we set significance to $p < 0.001$. For all subsequent assays, significance was considered $p < 0.05$.

Data Availability Statement:

Strains are available upon request. **APPENDIX F** contains detailed descriptions and sources of all strains used in this study.

Supplemental information: Supplemental information found online

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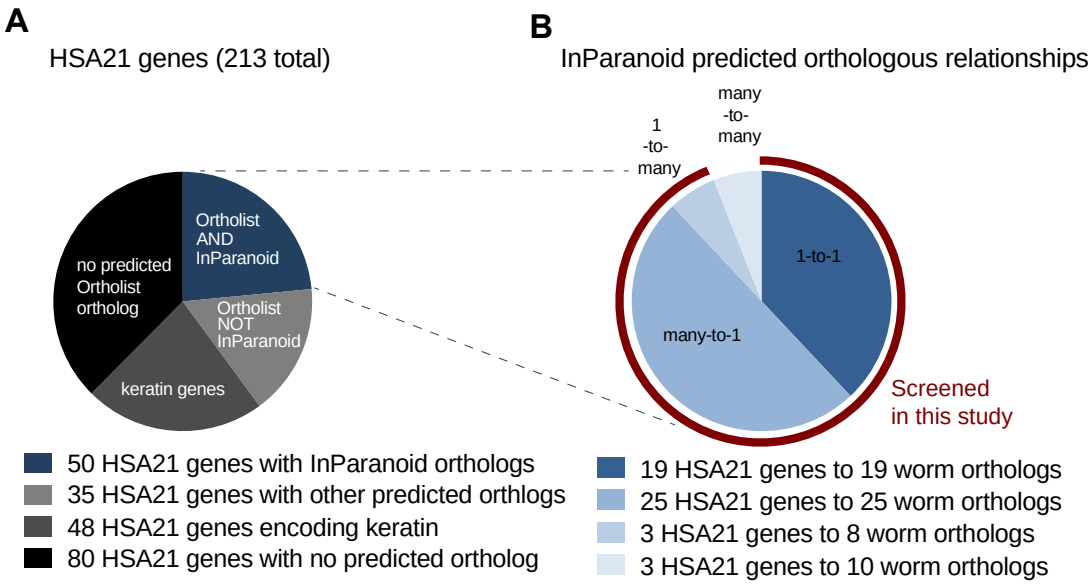

Figure 2.1: Representation of human 21st chromosome genes in *C. elegans*. (**A**) HSA21 encodes a conservative estimate of 213 protein-coding genes. Excluding the 48 genes predicted to encode keratin on HSA21 (*dark gray*), over half of the remaining 165 genes have predicted orthologs in *C. elegans* as identified by OrthoList. All 85 putative orthologs in worm (*light gray* and *blue*) with an available RNAi clone were tested in the RNAi viability screen (**APPENDIX B, D**). (**B**) Representation of InParanoid-predicted orthologs with their human to worm relationship (*shades of blue*). We tested all predicted InParanoid orthologs except those within a many-to-many relationship with RNAi for viability and mutants for behavioral and aldicarb assays (*red*).

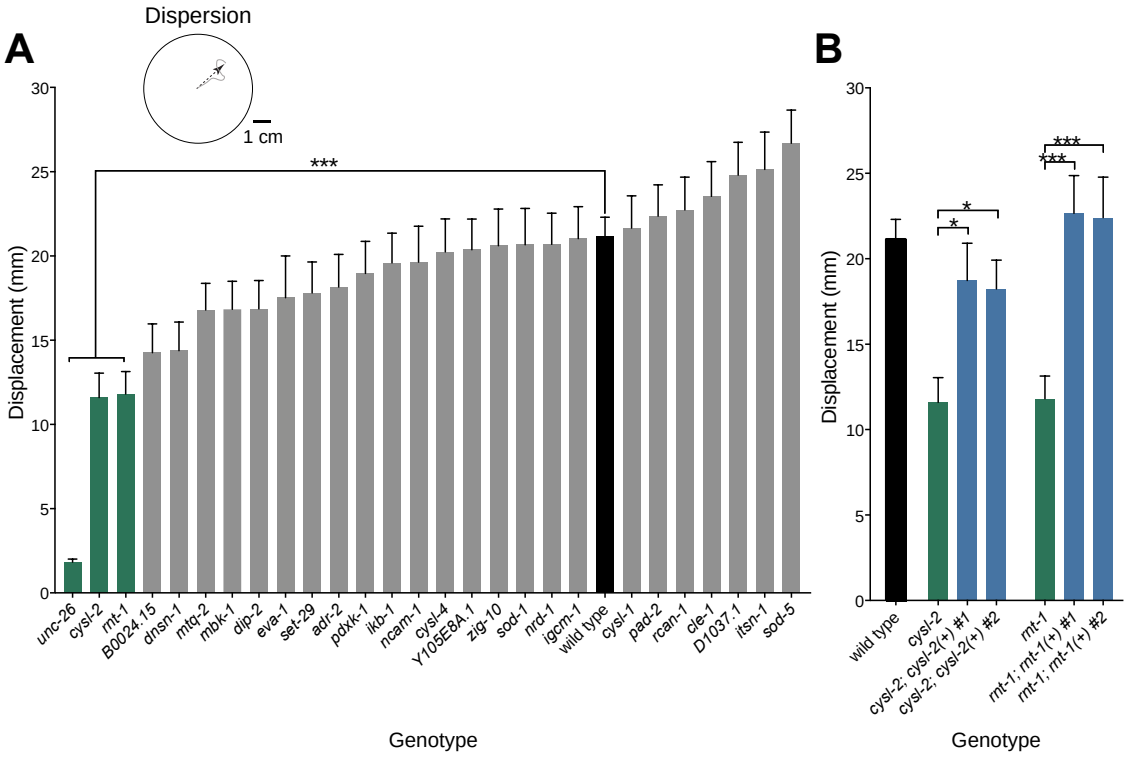

Figure 2.2: Screen for radial dispersion defects. Histograms show the mean displacement +/- SEM from start over ten minutes for each worm during a radial dispersion assay. **(A)** Three mutants (*green*) showed substantial reductions in dispersal relative to wild type (*black*). ***p < 0.001, n > 30. **(B)** Reductions in dispersal were rescued for the *cysl-2* and *mt-1* mutants (*green*) by extrachromosomal expression of the wild-type genes (*blue*). Wild-type performance shown for comparison (*black*). *p < 0.05, **p <0.01, ***p <0.001, n > 30.

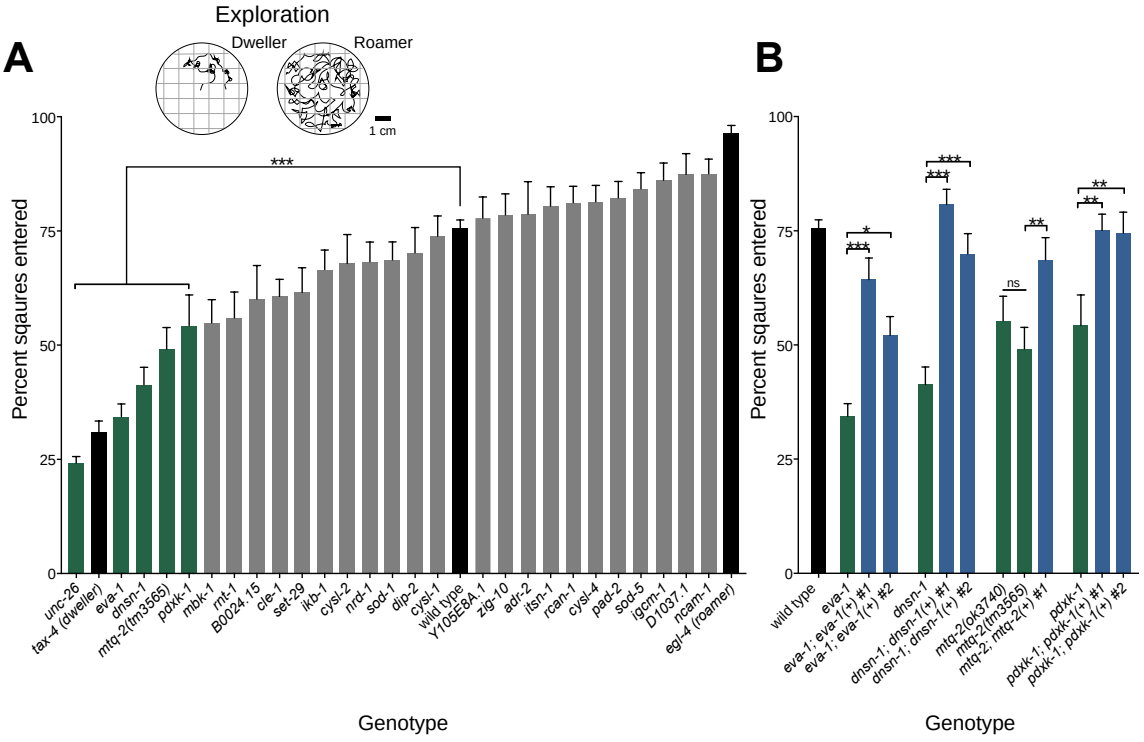

Figure 2.3: Screen for exploration defects. Histograms show the mean percentage of the assay plate traversed (percent of squares entered +/- SEM) by individual worms over a 16-h exploration assay. **(A)** Five mutants (*green*) covered significantly less area than wild type (*black*). ***$p < 0.001$, $n > 15$. **(B)** Reductions in exploration were rescued in the *eva-1*, *C24H12.5*, *mtq-2(tm3565)*, and *pdxk-1* mutants (*green*) by extrachromosomal expression of each of the wild-type genes (*blue*). Wild-type performance shown for comparison (*black*). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $n > 15$.

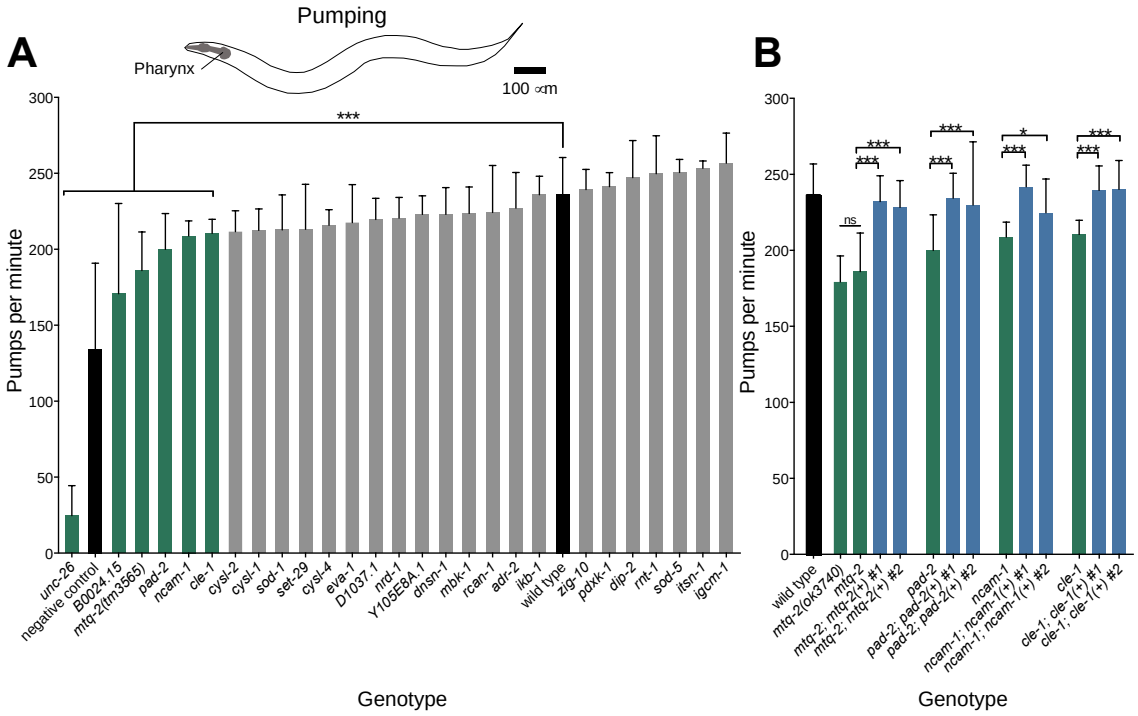

Figure 2.4: Screen for pumping defects. Histograms showing mean pharyngeal pumps per minute (+/- SD) in the presence of standard bacterial food. **(A)** Seven mutants (*green*) showed significant defects in pharyngeal pumping relative to wild type (*black*). ***p< 0.001, n > 30. **(B)** Mutants with pharyngeal pumping defects *mtq-2(tm3565), pad-2, ncam-1,* and *cle-1* (green) were rescued by extrachromosomal expression of the wild-type genes (*blue*). We did not observe rescue of *B0024.15*. *p < 0.05, **p < 0.01, ***p < 0.001, n > 30.

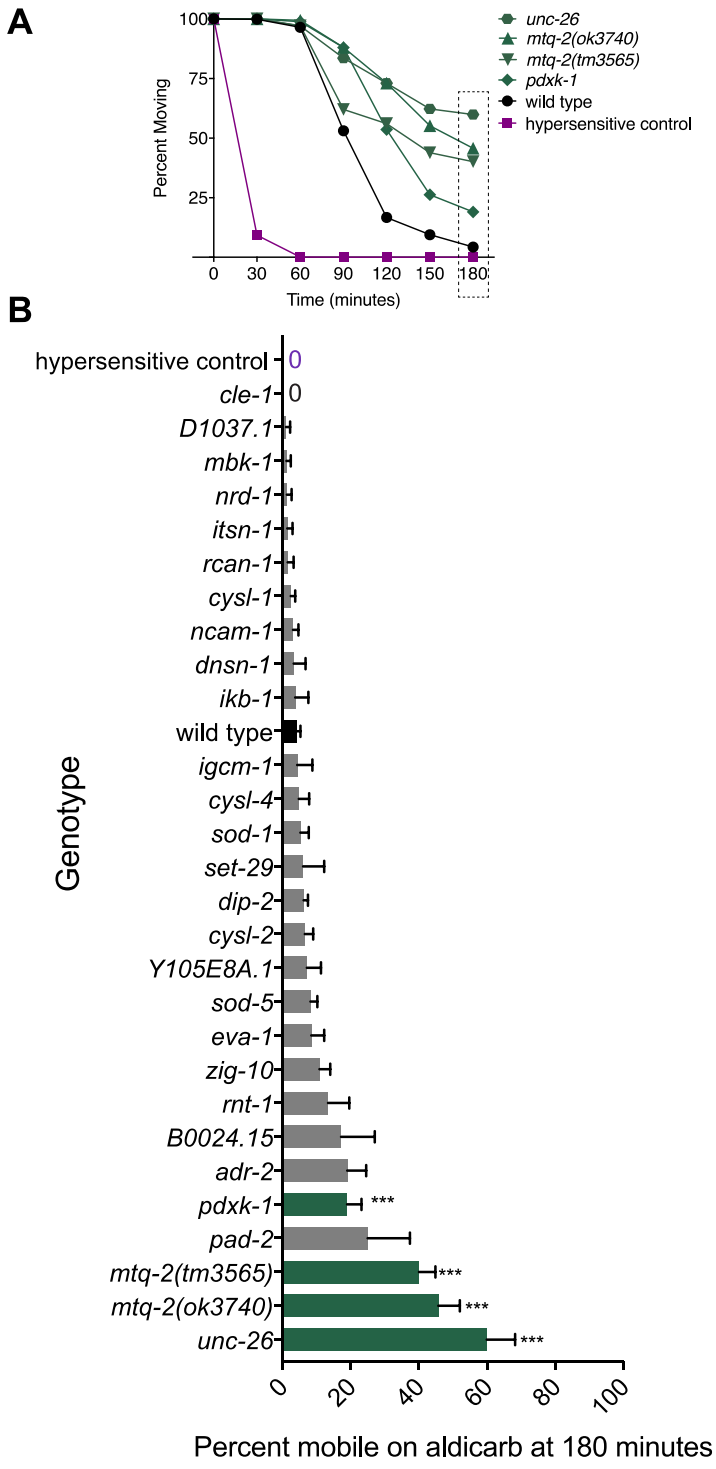

Figure 2.5: Screen for aldicarb resistance.

Figure 2.5: Screen for aldicarb resistance. We measured the full 180-minute time course to paralysis for 28 strains including wild type and hypersensitive control (*dgk-1*) in 1-mM aldicarb. Significant difference in paralysis rate was determined from WT with a two-way ANOVA using Dunnett's correction for multiple comparisons. No mutants showed significant hypersensitivity. **(A)** Mean percent (+/- SEM) of animals moving on aldicarb over minutes for WT, positive control, and mutants that were significantly different from WT: *pdxk-1(gk855208), mtq-2(tm3565), mtq-2(ok3740),* and *unc-26(e345)*. $p < 0.001$, $n > 3$. **(B)** Mean percent of (+/- SEM) of animals moving on aldicarb at the 180 minute time point for all mutants tested including those (*green*) that were significantly different from WT (*black*). Zero indicates that no animals were moving.

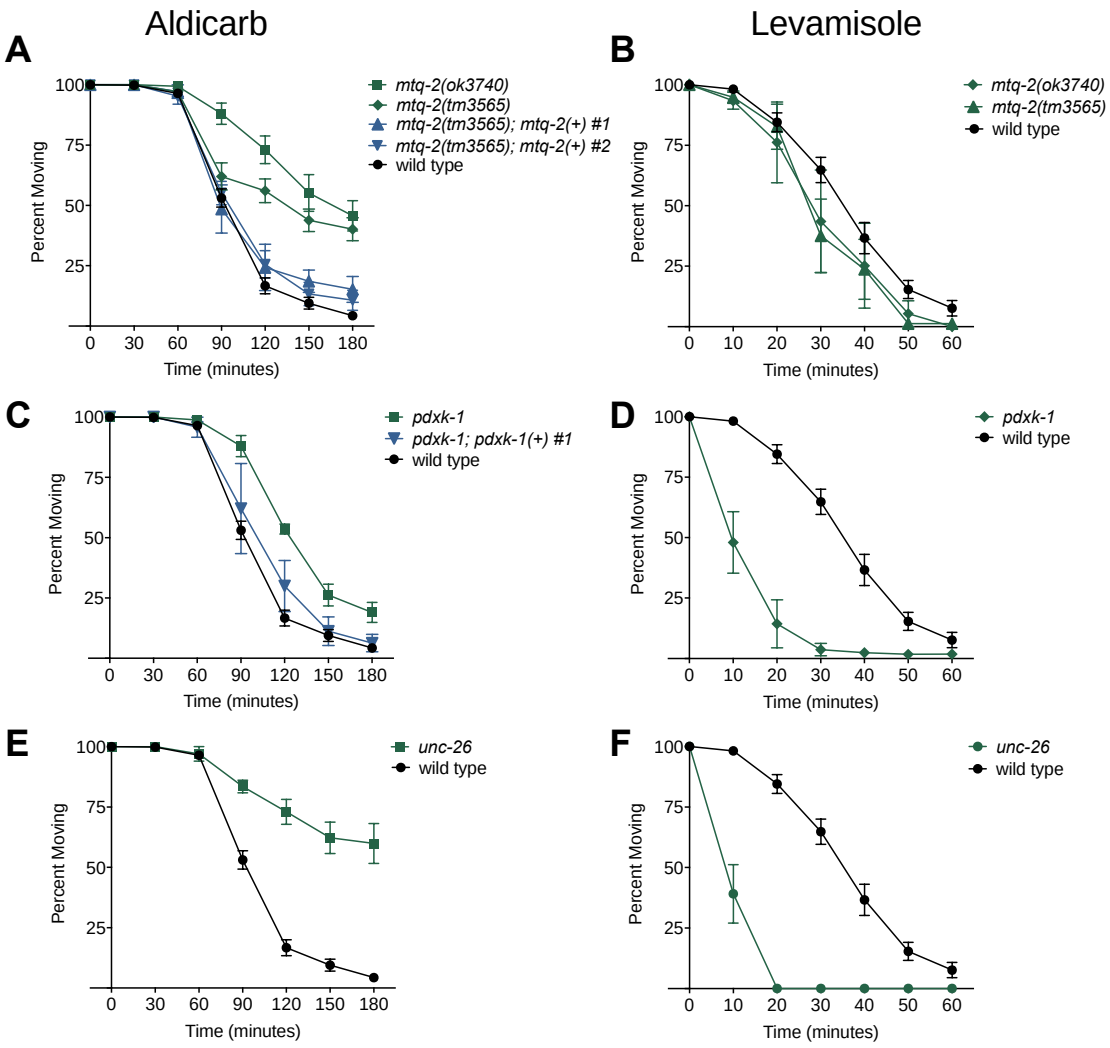

Figure 2.6. Response of deletion and putative loss-of-function mutants to aldicarb and levamisole.

Figure 2.6. Response of deletion and putative loss-of-function mutants to aldicarb and levamisole. Time course to paralysis on 1-mM aldicarb or 800-μM levamisole for aldicarb resistant mutants identified in screen (n > 3 trials, ~25 worms per trial). Wild-type controls were assayed in parallel. **(A)** Two independent alleles of *mtq-2* (*green*) displayed significant resistance to aldicarb, $p < 0.001$. Two of two rescue lines of *mtq-2(tm3565)* (*blue*) displayed significant sensitivity from the mutant background, $p < 0.05$. **(B)** Two independent alleles of *mtq-2* displayed sensitivity to levamisole. **(C)**. *pdxk-1(gk855208)* displayed significant resistance to aldicarb, $p < 0.001$. One of two rescue lines of *pdxk-1(gk855208)* (blue) was significantly improved from the mutant background, $p < 0.01$. **(D)** *pdxk-1(gk855208)* displayed hypersensitivity to levamisole relative to wild type, $p < 0.001$. **(E)** *unc-26(e345)* displayed significant resistance to aldicarb, $p < 0.001$ and **(F)** hypersensitivity to levamisole, $p < 0.001$.

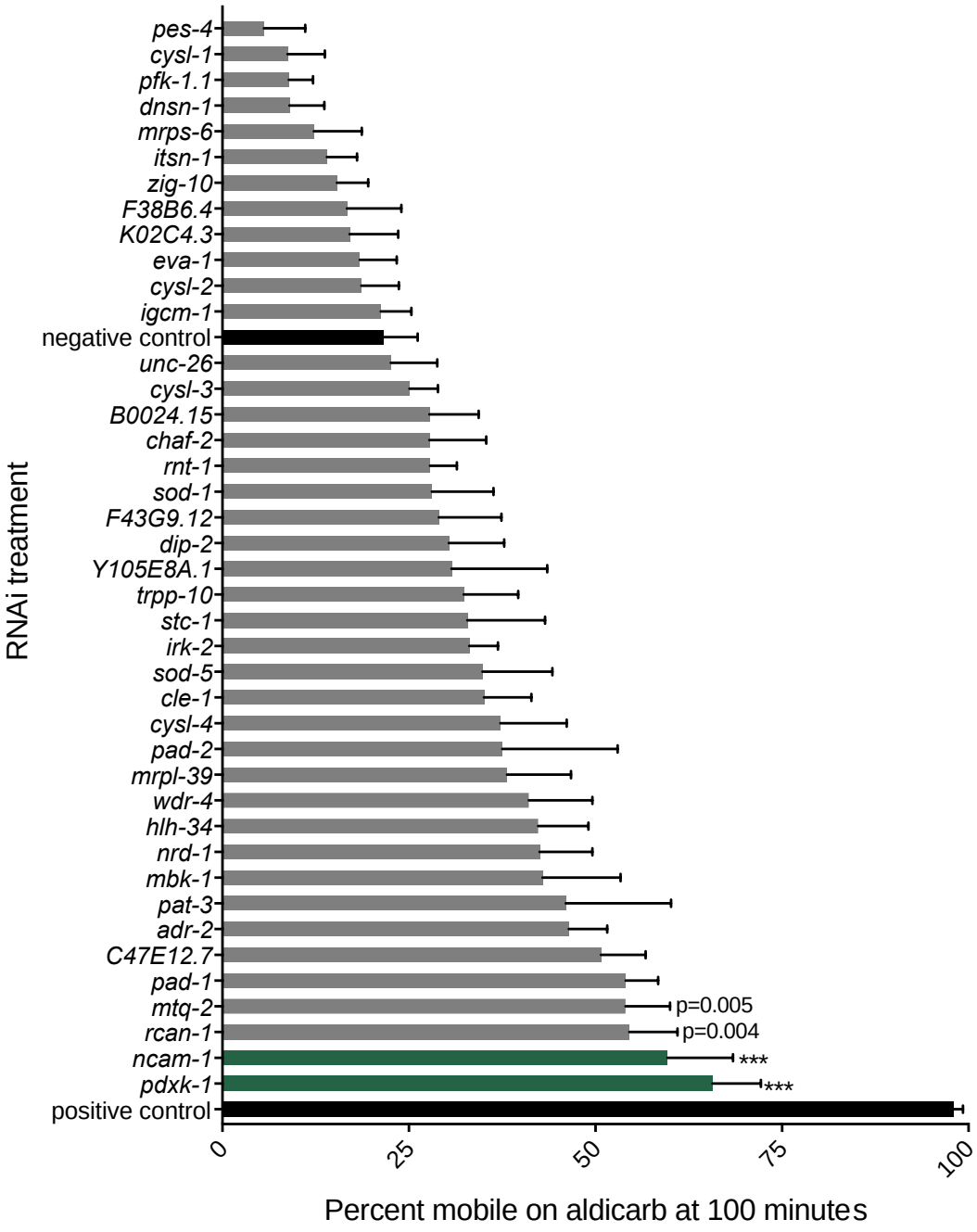

Figure 2.7. Aldicarb response following RNAi knockdown.

Figure 2.7. Aldicarb response following RNAi knockdown.
Aldicarb resistance of HSA21 orthologs in presence of RNAi treatment. RNAi treatment of *ncam-1* and *pdxk-1* genes (dark gray) resulted in significant resistance to paralysis by 1-mM aldicarb relative to the empty vector (L4440) negative control ($p < 0.001$, one-way ANOVA with Dunnet's post-hoc correction). Positive control was *egl-21*. RNAi of *unc-26* caused no change in sensitivity (not shown). RNAi-treatments were tested in triplicate over two generations when viable (n = 3-6 trials).

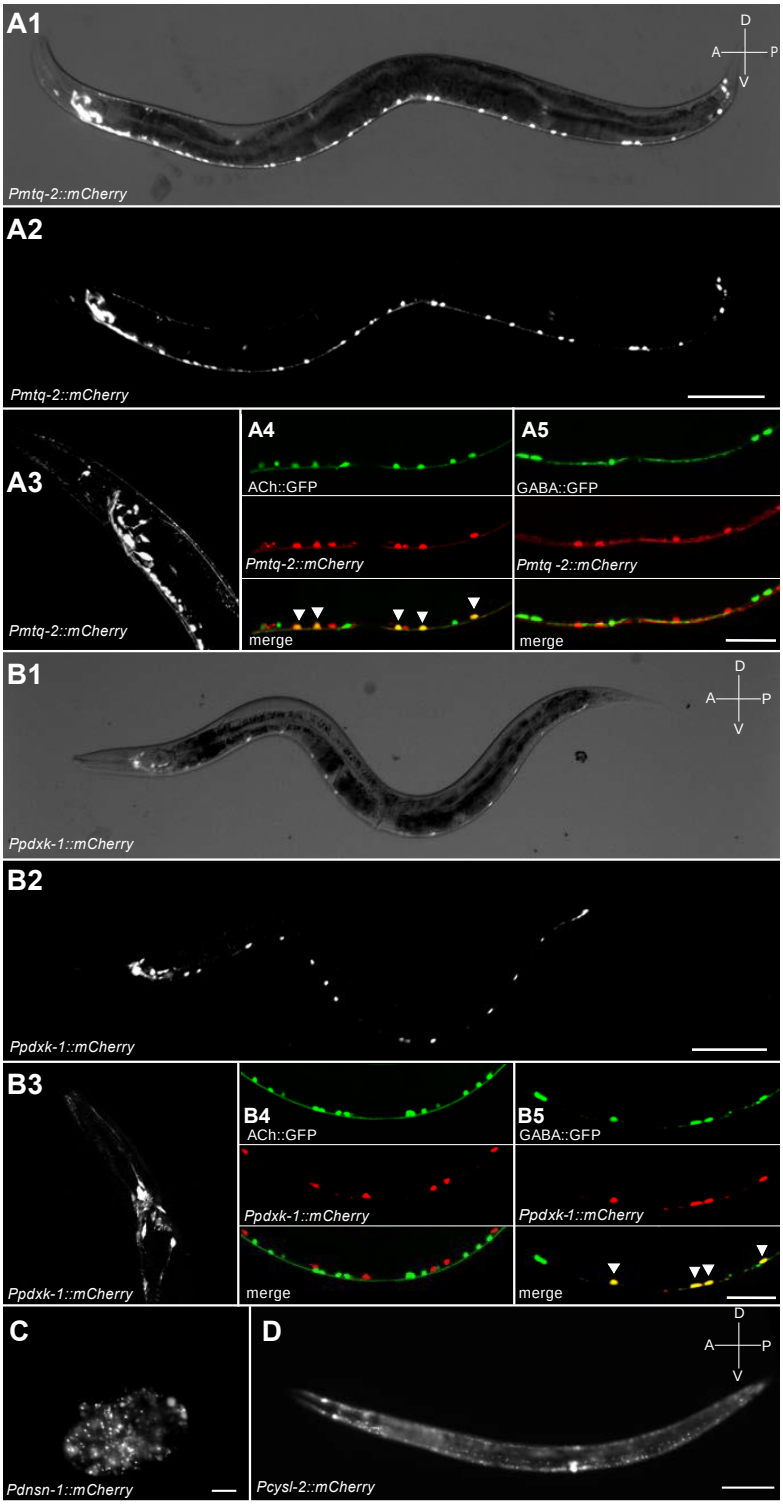

Figure 2.8. Expression pattern of select genes.

Figure 2.8. Expression pattern of select genes. Fluorescent micrographs of all HSA21 ortholog hits for which expression patterns were unknown. (**A1-3**) A transcriptional reporter for *mtq-2* expressed mCherry throughout the adult nervous system including in head (**A3**). Bright field image shown in **A1** for reference. Scale bar, 100 μm. Dual color images show co-expression of *mtq-2* reporter in cholinergic (*arrowheads*, **A4**) but not in GABAergic neurons (**A5**) in the ventral nerve cord. Scale bar, 50 μm. **(B1-3)** A transcriptional reporter for *pdxk-1* expressed mCherry throughout the adult nervous system including in head (**B3**). Bright field image shown in **B1** for reference. Scale bar, 100 μm. Dual color images show co-expression of *pdxk-1* reporter in GABAergic neurons (**B5**) but not cholinergic neurons (**B4**) in the ventral nerve cord. Scale bar, 50 μm. (**C**) A transcriptional reporter for *dnsn-1* expressed mCherry widely in developing embryo. Scale bar, 50 μm. (**D**) A transcriptional reporter for *cysl-2* expressed mCherry throughout body wall and vulval muscle. Scale bar, 100 μm.

Gene	Description	Behaviors				Expression			HSA21 Gene*	Mouse Gene*	Mouse Viability‡
		Dispersion	Exploration	Pumping	Aldicarb	Neuron	Muscle	Other			
cle-1	Collagen			↓		✓			*COL18A1*	*Col18a1*	viable
cysl-2	Cysteine synthase	↓					✓	✓	*CBS*	*Cbs*	postnatal lethal[1]
dnsn-1	Novel		↓					✓	*DONSON*	*Donson*	viable
eva-1	Slit receptor		↓			✓			*EVA1C*	*Eva1c*	viable
mtq-2	Glutamine methyltransferase		↓	↓	RIC	✓			*N6AMT1*	*N6amt1*	embryonic lethal[2]
ncam-1	Neural cell adhesion molecule			↓		✓			*NCAM2*	*Ncam2*	viable
pad-2	O-fucosyltransferase			↓		✓	✓		*POFUT2*	*Pofut2*	preweaning lethal
pdxk-1	Pyridoxal kinase		↓		RIC	✓			*PDXK*	*Pdxk*	preweaning lethal
rnt-1	Runx transcription factor	↓				✓	✓	✓	*RUNX1*	*Runx1*	embryonic lethal[3]
unc-26	Synaptojanin	↓	↓	↓	RIC	✓			*SYNJ1*	*Synj1*	perinatal lethal[4]

Table 2.1. Summary of HSA21 gene orthologs required for normal behaviors in *C. elegans*

↓ indicates the direction of changes that significantly differ from wild type ($p < 0.001$, one-way ANOVA)

RIC (Resistant to Inhibitors of Cholinesterase) indicates mutants with significant resistance to aldicarb *vs* wild type ($p < 0.001$, two-way ANOVA)

✓ indicates tissue(s) in which the gene is expressed. Data were derived from WormBase (Lee et al., 2018). Underline signifies results found in this study.

* HSA21 gene and mouse gene orthologs derived from InParanoid (Sonnhammer & Östlund, 2015).

‡ Mouse viability data derived from the Mouse Genome Informatics database (Blake et al., 2017).

Specific citations are as follows: [1](Watanabe et al., 1995), [2](P. Liu et al., 2010), [3](Okuda, Van Deursen, Hiebert, Grosveld, & Downing, 1996), [4](Cremona et al., 1999)

CHAPTER THREE

CHARACTERIZATION OF *MTQ-2*

ABSTRACT

Recently we identified *mtq-2* as a novel gene required for normal nervous system function and synaptic transmission in a screen of human 21st chromosome orthologs in *C. elegans*. Here, we use behavioral genetics and confocal microscopy to functionally characterize *mtq-2*, which, encodes a well-conserved ortholog of human *N6AMT1*, a predicted glutamine methyltransferase. The phenotypes of *mtq-2* mutants are reminiscent of Gα signaling mutants, which prompted us to investigate potential interactions among genes in this signaling pathway. We found that mutants of the Gα_o subunit GOA-1 strongly suppressed *mtq-2* loss-of-function phenotypes, suggesting that *mtq-2* may act upstream of or in conjunction with *goa-1* to mediate synaptic vesicle release. As a novel molecular modulator of neurotransmission, *mtq-2* may contribute to neurological phenotypes when overexpressed in Down syndrome and should be prioritized for further study.

INTRODUCTION

Faithful signal transduction at the chemical synapse requires precise spatiotemporal coordination of hundreds of molecules. Proteins involved in synaptic vesicle release perform diverse functions, from comprising the core synaptic vesicle machinery to modulating levels of second messenger molecules. Among the latter class, heterotrimeric G proteins have been among the most intensively studied. In addition to regulating levels of second messenger molecules at the synapse, G proteins are also involved in many aspects of development. With such myriad and important functions, it is unsurprising that G proteins—the Gα subunit in particular—are subject to many forms of regulation. Gα proteins are molecular switches, activated, or "on" in the presence of GTP and inactivated, or "off," when bound to GDP. Thus, regulation of G proteins often revolves around control of the GTP or GDP bound state. There are three broad categories of proteins that regulate the duration of the Gα signal: 1) RGS (regulators of G protein signaling) proteins rapidly terminate the Gα signal by promoting the hydrolysis of GTP for GDP; 2) GEFs (guanine nucleotide exchange factors) stimulate the exchange of GDP for GTP thereby activating the Gα protein; and 3) GDIs (guanine nucleotide dissociation inhibitors) block the exchange of GDP for GTP and lock the Gα protein in its inactive state (Schmidt & Hall, 2002). Finally, heterotrimeric Gα proteins can be post-translationally modified. For instance, lipidation of select residues on Gα proteins encourages appropriate localization to microdomains within the cell (Schmidt & Hall, 2002).

In a previous screen of human 21st chromosome orthologs in *C. elegans*, we identified a novel gene, *mtq-2 (methyltransferase modifying glutamine (Q)*, required

for multiple behaviors and normal synaptic signaling (**Table 3.1**). Though this gene is well-conserved, its functional role is unclear. Mice lacking the orthologous gene, *N6amt1*, die very early in embryonic development, precluding further study (P. Liu et al., 2010). In contrast, *C. elegans mtq-2* mutants are viable.

By homology, *mtq-2* is a glutamine methyltransferase. In general, glutamine methylation is less well-characterized than methylation of arginine or lysine residues; nevertheless, non-histone glutamine methylation does occur on translation termination factors and ribosomal proteins (Polevoda & Sherman, 2007). Direct evidence for glutamine methylation by MTQ-2 on class I translation termination factors (ETF1 in eukaryotes and prmC in bacteria) has been shown in *E. coli*, yeast, and mouse (P. Liu et al., 2010; Nakahigashi et al., 2002; Polevoda et al., 2006). Biochemically, methylation of the target glutamine on the release factor is associated with increased efficiency of translation termination either by directly participating in hydrolysis of the tRNA-peptide bond or by increasing the affinity of the release factor to the ribosome (Dinçbas-Renqvist et al., 2000; Korostelev, Zhu, Asahara, & Noller, 2010; H. Song et al., 2000). More recently, a minimal recognition motif for MTQ-2 was discovered, GQX_3R , and the MTQ-2 complex was found to methylate hundreds of other substrates *in vitro* (Kusevic et al., 2016). This raises the possibility that MTQ-2 may have targets other than the translation termination factor.

The biochemical evidence that MTQ-2 may bind additional substrates, coupled with the behavioral phenotype of *mtq-2* we previously reported (**Table 3.1**), suggested that *mtq-2* may be interacting with one or more proteins already known to mediate synaptic signaling in worm. In particular, *mtq-2* mutants were

phenotypically reminiscent of mutants involved in Gα signaling. Moreover, almost all *C. elegans* Gα proteins contain the *mtq-2* minimal recognition motif, and this motif is conserved across diverse species in Gαo (**Figure 3.1**). Here we show that *mtq-2* is required for normal nervous system function, normal excitatory neurotransmission, and that it functions, specifically, in cholinergic neurons to mediate its effects. Finally, we provide evidence of a potential genetic interaction between *mtq-2* and *goa-1* that future research should seek to confirm.

RESULTS

mtq-2 functions in cholinergic neurons to mediate neurotransmission

We initially identified *mtq-2* as a gene required for several behaviors dependent on proper nervous system function (**Table 3.1**). Further, we found that a transcriptional reporter of *mtq-2* expressed robustly and exclusively in neurons and, specifically, cholinergic neurons. Given the dependency of many of the behaviors on cholinergic function and the expression pattern of *mtq-2*, we tested whether a cholinergic promoter driving *mtq-2* was sufficient to restore function of the aldicarb response to wild-type levels. We found that cholinergic expression of *mtq-2* in a null background restored aldicarb sensitivity to wild-type levels (**Figure 3.2**). This suggests that *mtq-2* functions largely in cholinergic neurons to mediate neurotransmission. This interpretation is strengthened by our previous finding that *mtq-2* showed wild-type sensitivity to the nAChr agonist levamisole (Nordquist et al., 2018)

MTQ-2 localizes to cholinergic synapses and processes

To determine the subcellular localization of MTQ-2, we attached mCherry to the C-terminus of genomic MTQ-2, while preserving the endogenous 5′ and 3′ regulatory elements. We found that MTQ-2::mCherry localized to cholinergic axons and presynapses indicating that MTQ-2 may play more of a direct role in synaptic vesicle release (**Figure 3.3**). To ensure that the fluorescent tag was not disrupting the function of the native protein, we performed an aldicarb assay and observed full rescue of the loss-of-function phenotypes with MTQ-2::mCherry (**Figure 3.3**). Together, these findings are consistent with a role for *mtq-2* in regulating synaptic release at cholinergic synapses.

MTQ-2 does not strongly affect the development of synapses

Deficiencies in cholinergic release could be caused by a failure of cholinergic synaptic formation or localization or by decreased release in existing cholinergic synapses (Hiley, McMullan, & Nurrish, 2006). We were curious if *mtq-2* caused deficiencies in synaptic vesicle release due to fewer synapses or due to deficiencies in release at existing synapses. To address the former point, we examined the number, distribution, and relative fluorescent intensity of cholinergic synapses (Klassen & Shen, 2007). In worm, synapses form *en passant* along the dorsal cord axon in a highly-stereotyped manner and can be visualized with a synaptic vesicle associated marker (Klassen & Shen, 2007). Fluorescent reporters provide a readily observable measure of synapse development and/or functional state. Each punctum corresponds to a single neuromuscular junction (Jin, Jorgensen, Hartwieg, & Horvitz, 1999). Further, electron microscopy experiments have revealed a correlation between the relative intensity of the

synaptic vesicle reporter and the number of synaptic vesicles (Richmond & Jorgensen, 1999; Weimer et al., 2003).

As a model of synaptic number and intensity, we examined synaptic puncta in the cholinergic DA9 motoneuron. The DA9 neuron is one of the most posteriorly located neurons in the worm. DA9 extends a dendritic process anteriorly along the ventral cord and sends a commissural axon to the dorsal cord (**Figure 3.4)**. Adult wild-type animals have around 25 synapses along the length of the axonal dorsal cord. We found no differences between *mtq-2* mutants and the control strain in the number of cholinergic synapses, their distribution along the dorsal cord axon, or their relative fluorescent intensity (**Figure 3.5**). In *mtq-2* mutants, we did observe ectopic puncta in the dendrite; however, this phenotype was of low penetrance (1 of 10 animals), and we did not pursue it further. These results most plausibly suggest that *mtq-2* may affect release dynamics of neurotransmitter from preexisting synapses, yet we cannot conclusively rule out a developmental role.

mtq-2 may act upstream of or in conjunction with goa-1

The phenotypes of *mtq-2* are similar to those of other mutants involved in Gα signaling and prompted us to explore whether *mtq-2* was interacting with genes in these pathways to regulate synaptic vesicle release. There are three main classes of heterotrimeric G proteins known to be involved in neurotransmitter release: Gα_O, Gα_Q, and Gα_S; all of which are represented by one ortholog of each class in worm (Bastiani & Mendel, 2006; Brown & Sihra, 2008). Both *gsa-1* (Gα_S) and *egl-30* (Gα_Q) inhibit neurotransmitter release, while *goa-1* (Gα_O) promotes

neurotransmitter release, primarily through inhibition of *egl-30* ($G\alpha_Q$) (**Figure 3.7)**.

Worm has been a particularly informative system for elucidating the function of G proteins; in part, because of the readily observable phenotypes of the Gα mutants (Koelle, 2016). Loss of function mutants of $G\alpha_s$ and $G\alpha_q$ are characteristically sluggish, egg retentive, and resistant to the acetylcholinesterase inhibitor aldicarb. Loss of function mutants of $G\alpha_O$ display the converse phenotypes: locomotor hyperactivity, constitutive egg laying, and hypersensitivity to the acetylcholinesterase inhibitor aldicarb (Koelle, 2016).

Given the opposite loss-of-function phenotypes for mutants of *goa-1* ($G\alpha_O$) and *mtq-2*, we focused on *goa-1* for epistasis analysis. If *mtq-2* functions upstream of *goa-1*, we would expect the double mutant *mtq-2; goa-1* to show the phenotypes of the *goa-1* single mutant (i.e. aldicarb hypersensitivity and constitutive egg laying). We found that the double mutant of *mtq-2; goa-1* strikingly suppressed the *mtq-2* phenotypes suggesting that *mtq-2* functions upstream of or in conjunction with *goa-1* (**Figure 3.6**).

goa-1 mediates synaptic vesicle release via two known pathways: a *dgk-1* (diacylglycerol kinase) dependent pathway and an *eat-16* (RGS) dependent pathway. Importantly, both *dgk-1* and *eat-16* were found to function downstream of *goa-1* in a genetic screen for suppressors of activated *goa-1* (Hajdu-Cronin, Chen, Patikoglou, Koelle, & Sternberg, 1999). It is currently unknown if *goa-1* directly binds *dgk-1* and *eat-16* or if intermediate targets exist; biochemical studies have yet to confirm these genetic relationships (Bastiani & Mendel, 2006). Nevertheless, *dgk-1* assumes a critical role at the synapse. A kinase, *dgk-1*

phosphorylates the second messenger molecule DAG (diacylglycerol), thereby converting it to PA (phosphatidic acid). Levels of DAG within the cell directly correlate to levels of synaptic vesicle release, and, accordingly, loss of function mutants of *dgk-1* are extremely hypersensitive to the paralytic effects of aldicarb (van Swinderen et al., 2001). We examined the time course to paralysis for *mtq-2; dgk-1* double mutants and found that they displayed intermediary egg-laying and aldicarb response phenotypes of *dgk-1* and *mtq-2* single mutants. Qualitatively, *mtq-2; dgk-1* double mutants also displayed a mixture of phenotypes from the *mtq-2* and *dgk-1* single mutants: their crawl speed was intermediate to the single mutants, yet they moved with the characteristic jerkiness of *dgk-1* **(Fig 3.6)**. These results most likely suggest that *mtq-2* acts upstream of *goa-1* to mediate SV release via diacylglycerol kinase *dgk-1* independent genetic pathway.

DISCUSSION

Here we characterize a novel gene, *mtq-2*, which we found to mediate neurotransmitter release from cholinergic neurons in *C. elegans*. We further show that loss of *mtq-2* predominantly disrupts the function of preexisting synapses in the adult animal. Finally, we discovered that loss of *goa-1* strikingly suppressed *mtq-2* phenotypes, which suggests that *mtq-2* functions upstream of or in conjunction with *goa-1* to regulate synaptic vesicle release from cholinergic neurons.

The presence and high conservation of the recently discovered *mtq-2* minimal recognition motif among Gα proteins—including GOA-1 (Gαo)—is

intriguing and suggests that MTQ-2 may regulate GOA-1 activity by direct methylation. On the human ortholog of *goa-1* (*GNAO1*), the target glutamine (Q205) resides within a highly conserved nucleotide binding pocket (Mendel et al., 1995). Previous studies have revealed that a Q205L substitution constitutively activates Gαo, presumably by blocking GTPase activity and locking the Gα protein in its activated state (Slepak, Wilkie, & Simon, 1993; Strittmatter, Fishman, & Zhu, 1994). It is tempting to speculate that methylation of Q205 may somehow interfere with GDP to GTP exchange; however, additional research will first need to determine the presence of methylglutamine on Gαo.

In summary, our findings provide initial support for a novel MTQ-2-mediated mechanism for the influence of synaptic function. Further research will be necessary to evaluate whether MTQ-2 methylation of Gα proteins underlies this potentially conserved pathway.

MATERIALS AND METHODS

Strains

C. elegans were grown on NGM (nematode growth media) agar plates seeded with OP50 bacteria at 20 °C as described (Brenner, 1974). N2 Bristol was wild type and was also used for outcrossing. The following mutant strains were used is this study: JT734 *goa-1(sa734)* I; VC3037 *mtq-2(ok3740)* II; FX3565 *mtq-2(tm3565)* II; PS2627 *dgk-1(sy428)* X; JPS1016 *mtq-2(ok3740)* II; *goa-1(sa734)* I; JPS1014 *mtq-2(ok3740* II*); dgk-1(sy428)* X.

Transgenic strains

The following transgenic strains were used in this study: *wyIs85 (Pitr-1 pB::GFP:: rab-3)* was used as a reporter and to generate JPS787 *(mtq-2(ok3740 II; wyIs85 (Pitr-1 pB::GFP:: rab-3))*. The deletion allele *mtq-2(ok3740) II* was confirmed with standard genotyping procedures. The following strains express *mtq-2* under the *unc-17* promoter: JPS1080 *vxEx1080[Punc-17::MTQ-2::mtq-2UTR, pmyo-2::mCherry]; mtq-2(ok3740)II*, JPS1081 *vxEx1081[Punc-17::MTQ-2::mtq-2UTR, pmyo-2::mCherry]; mtq-2(ok3740)II*, JPS1082 *vxEx1082[Punc-17::MTQ-2::mtq-2UTR, pmyo-2::mCherry]; mtq-2(ok3740)II*, JPS1083 *vxEx1083[Punc-17::MTQ-2::mtq-2UTR, pmyo-2::mCherry]; mtq-2(ok3740)II*. To generate strains expressing MTQ-2 tissue-specifically, we first subcloned MTQ-2 into a pCR™-Blunt II-TOPO® vector and confirmed the absence of mutations by sequencing. We used SphI and BamHI to pull out the *unc-17* promoter from the PT63 plasmid and used Gibson Assembly® to stitch the *unc-17* promoter and MTQ-2 gene and 3' UTR fragments together. The following strain is mCherry tagged MTQ-2: JPS836 *vxEx836[pmtq-2::MTQ-2::mCherry::mtq-2 UTR, coel::GFP]; mtq-2(ok3740) II*. We also used Gibson Assembly® to attach mCherry to the C-terminus of MTQ-2. The mCherry fluorophore was derived from pCFJ90.

Aldicarb assay

Aldicarb assay were performed as described in (Mahoney et al., 2006). We first prepared 1mM aldicarb plates by dissolving an aldicarb stock solution into cooled, standard NGM agar. Plates were left to dry on the bench overnight before placing in a 4°C incubator. On the day of the assay, plates were removed

an allowed to come to room temperature for about 30 minutes. 25-30 staged day one adults were transferred from a seeded OP50 plate to the center of the aldicarb treated plate. We transferred animals in the presence of food to encourage animals to remain in the center of the plate. During the first hour of the assay, animals were gently moved back to the center of the plate to facilitate scoring. Each half hour, an individual animal was observed for spontaneous movement on an Olympus SZX16 Stereo Microscope. If the animal had ceased spontaneous movement, we gently prodded its head and tail three times to determine if the animals responded to evoked touch. Animals were considered paralyzed when no movement was observed following gentle prodding.

Egg retention assay

The assay for egg retention was performed essentially as described in (Hart, 2006). 24 hours (± 1 hour) before the start of the experiment, late L4 animals were transferred to a new, seeded plate and allowed to develop to adulthood at room temperature. The day of the experiment, a 10% bleach solution was freshly prepared and passed through a syringe filter (VWR International 25mm syringe filter with 0.2 μm nylon membrane, 28145-147) to remove debris. 50 μL was pipetted into individual wells of a 96 well plate. A single animal was picked into a single well and incubated until its cuticle was well-dissolved (8-20 minutes). The number of eggs per animal was counted on an Olympus SZX16 Stereo Microscope. Only animals with fully dissolved cuticles were scored. Care was taken to ensure each animal was positioned in the center of the well. Any animal not positioned in the center of the well was not scored

due to the difficulty of accurately resolving egg number on the edges of the well. Assays were performed blind and repeated on at least three separate days.

Confocal microscopy

Staged day one adult animals were placed on an agarose pad and immobilized with NaN_3. All animals were imaged within 45 minutes of mounting. We used a Leica Plan-Apochromat 63X/1.4 objective on a Leica DM6000 CFS confocal microscope to collect z-stacks of the dorsal nerve cord. For all experiments, FIJI was used to create maximum z-stack projections of the dorsal cord of each animal. For experiments measuring synapse localization, puncta count, and relative fluorescent intensity, gain and offset settings were invariant. To determine puncta count, we set a threshold based on the wild-type reporter strain and used the "Analyze Particles" plug-in in FIJI. To determine relative fluorescent intensity along the nerve cord, we isolated ten dorsal nerve cords from each animal. We defined the bend at which the commissural axon curves into the dorsal cord as the starting point (i.e. 0 on x-axis) and extended s line 175 μm out. We used the "Straighten" plug-in in FIJI to straighten the dorsal nerve cords and the "Plot Profile" plug-in to generate fluorescent intensity as a function of distance for the composite images.

Statistical analysis

For radial dispersion, exploration, pharyngeal pumping, and egg retention assays, we compared values to the wild-type control with a one-way ANOVA with a Bonferroni correction to evaluate select comparisons or

Dunnett's test when all comparisons were to wild type. Aldicarb responses of mutants were compared to wild-type control with a two-way ANOVA and a Bonferroni correction for select comparisons or Dunnet's test when all comparisons were to wild type. We used GraphPad Prism7 software. Significance was considered $p < 0.05$.

ACKNOWLEDGEMENTS

We thank Kang Shen for the DA9 reporter strain and Derek Sieburth for the plasmid PT63, which contained the *unc-17* promoter used for cloning. Luisa Scott offered constructive feedback and edits.

Genotype	Aldicarb response[1, 2]	Levamisole response[1]	Eggs *in utero*[2]
N2	WT	WT	13 ± 0.4
mtq-2(tm3565)	RIC	WT	20 ± 1.4
mtq-2(tm3565); mtq-2(+)	WT	-	-
mtq-2(ok3740)	RIC	WT	22 ± 0.7
mtq-2(ok3740); mtq-2(+)	WT	-	16 ± 1

Table 3.1. Summary of phenotypes of *mtq-2*
Dashes indicate no data.
[1] indicates data previously published in (Nordquist et al., 2018).
[2] indicates data collected in this study
Mean egg in utero presented ± s.e.m. JPS837 is rescue strain for *mtq-2(ok3740); mtq-2(+)*

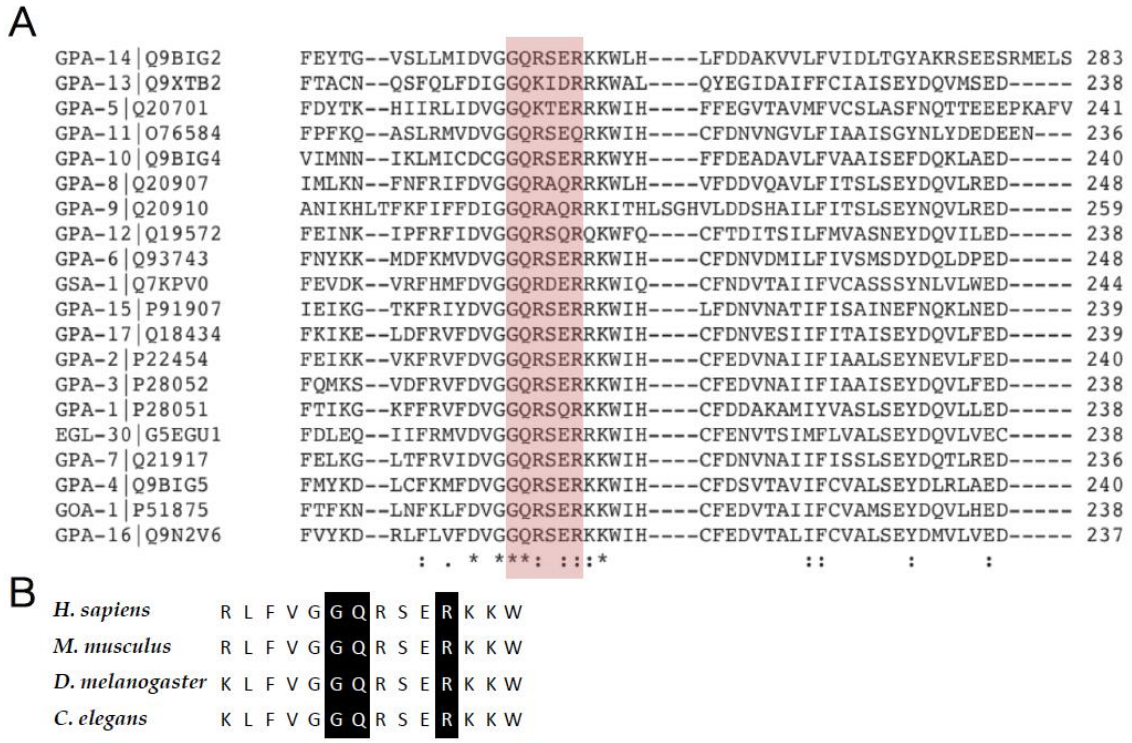

Figure 3.1. Conservation of *mtq*-2 minimal recognition motif in Gα proteins. **A.** Alignment of all Gα proteins in *C. elegans* (Bastiani & Mendel, 2006). The *mtq-2* minimal recognition motif (Kusevic et al., 2016) is highlighted in red. **B.** Alignment Gα_o across species.

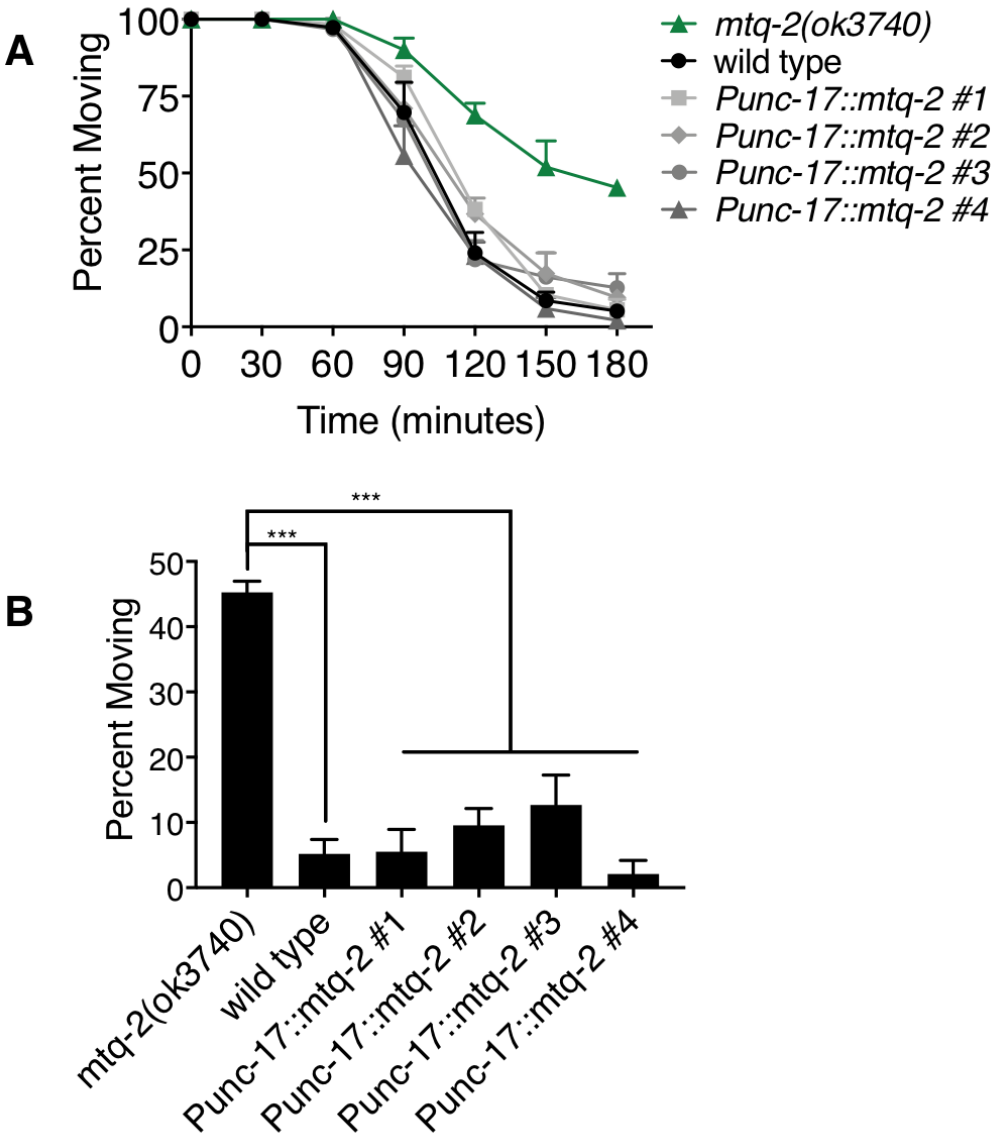

Figure 3.2. *mtq-2* functions in cholinergic neurons to mediate aldicarb response

Shown here is the (**A**) full time course to paralysis in the presence of 1-mM aldicarb and (**B**) response to aldicarb at 180 minutes plotted for clarity. Mean percent of responsive animals are plotted. Error bars indicate s.e.m. Trials for all strains were performed at least three times.

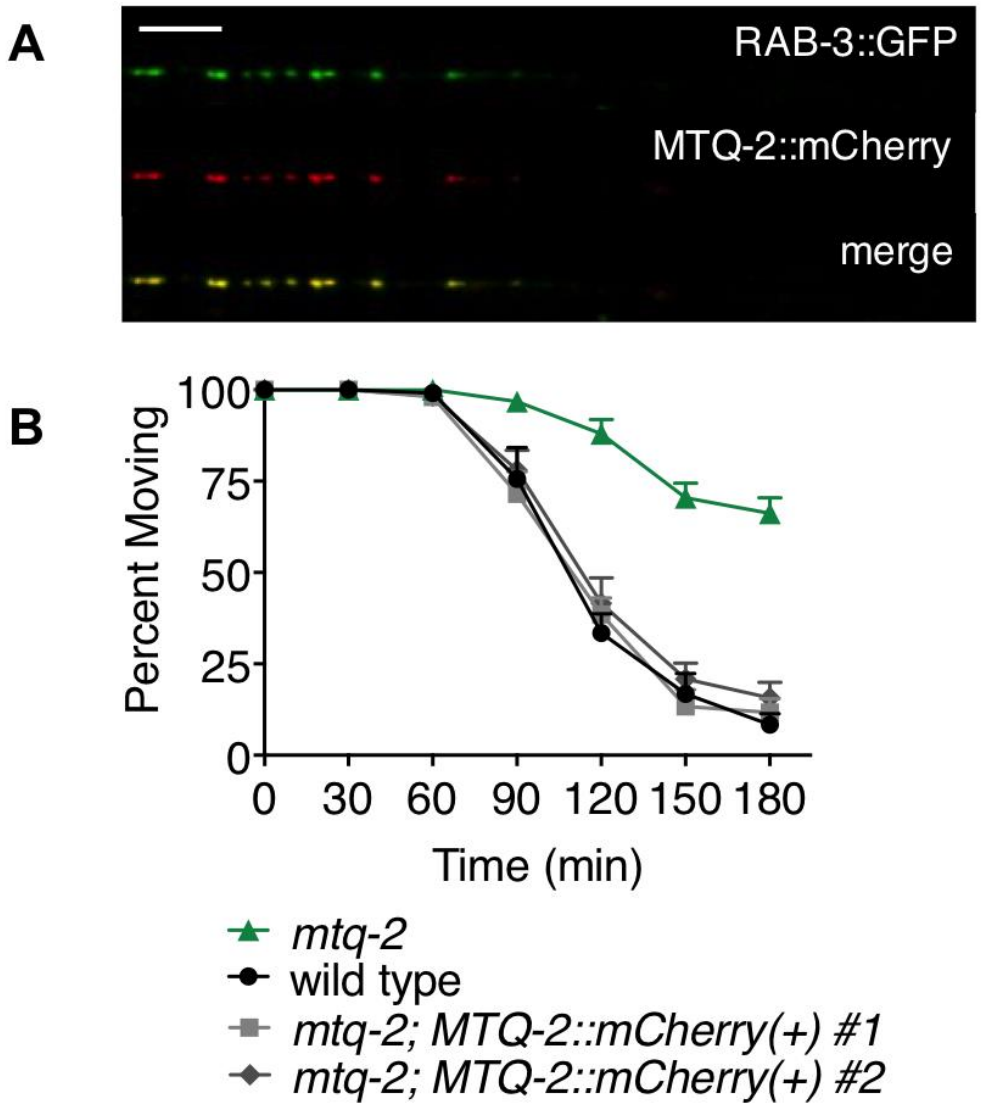

Figure 3.3. MTQ-2::mCherry localizes to cholinergic synapses
A. Confocal micrographs showing RAB-3::GFP, MTQ-2::mCherry, and merged synapses of the cholinergic DA9 neuron along the dorsal cord. All animals are oriented such that left is anterior and up is dorsal. Scale bar, 10 μm. **B.** mCherry tagged MTQ-2 rescues aldicarb response of *mtq-2(ok3740)* indicating that mCherry does not interfere with the normal function of the protein. Mean percent of responsive animals shown. Error bars are s.e.m. At least five trials were performed for each strain.

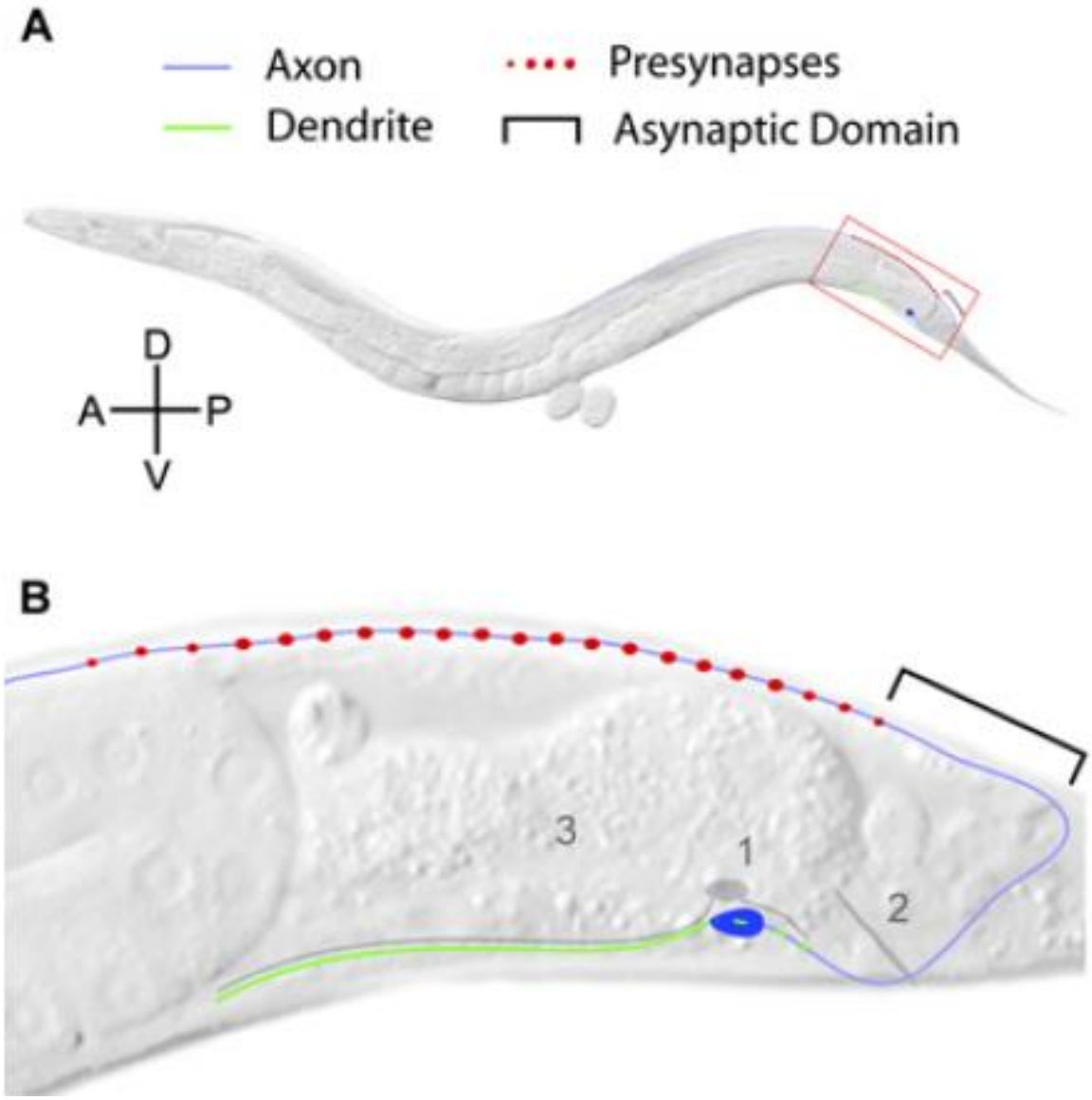

Figure 3.4. Location of DA9 neuron and axonal puncta
A. Red box indicates location of the DA9 cholinergic neuron in the tail of the animal. **B.** Enlargement of area circumscribed in **A**. Numbers refer to other anatomical landmarks: 1) VA12 neuron, 2) anus, 3) gut. The soma and axon of DA9 is shown in blue. The DA9 dendrite is shown in green. The stereotyped location of DA9 synaptic puncta are shown as red dots along the dorsal cord. Bracket indicates asynaptic region. From (Klassen & Shen, 2007)

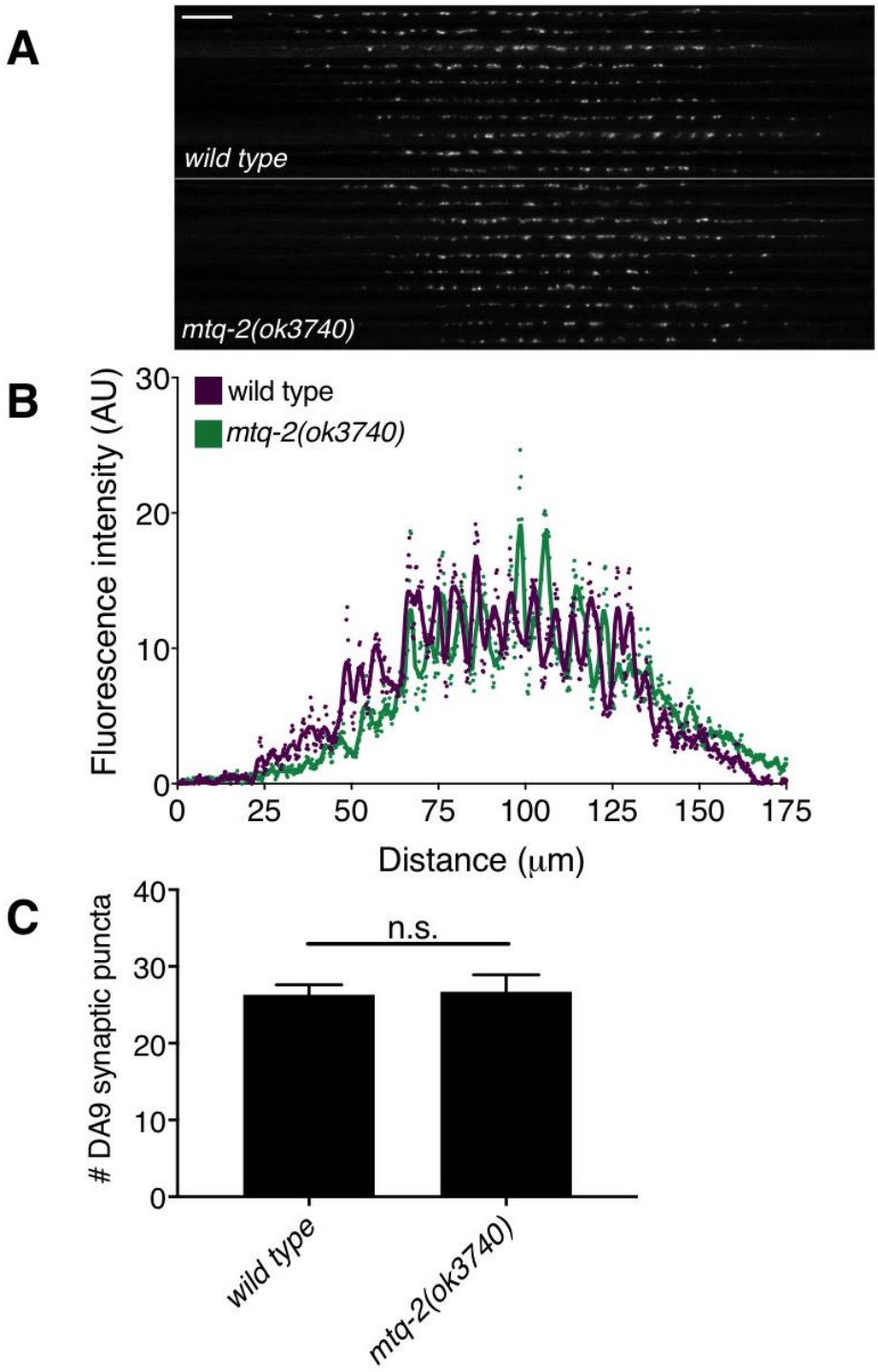

Figure 3.5. *mtq*-2 does not alter synaptic vesicle localization or fluorescence intensity in the DA9 motoneuron.

Figure 3.5. *mtq*-2 does not alter synaptic vesicle localization or fluorescence intensity in the DA9 motoneuron. A. Confocal micrographs of ten straightened and aligned dorsal cords from wild-type animals and *mtq-2(ok3740)* animals. All animals are oriented such that let is anterior and up is dorsal. Scale bar, 10 μm. **B.** Quantification of fluorescence intensity for composite images. Wild-type animals are shown in purple and *mtq-2(ok3740)* in green. Dotted lines represented actual values and solid lines a 10 μm sliding average. **C.** Average number of synaptic puncta per genotype. There are no differences ($p > 0.05$) in the mean number of synaptic puncta between wild-type and *mtq-2 (ok3740)*. Error bars indicate s.e.m.

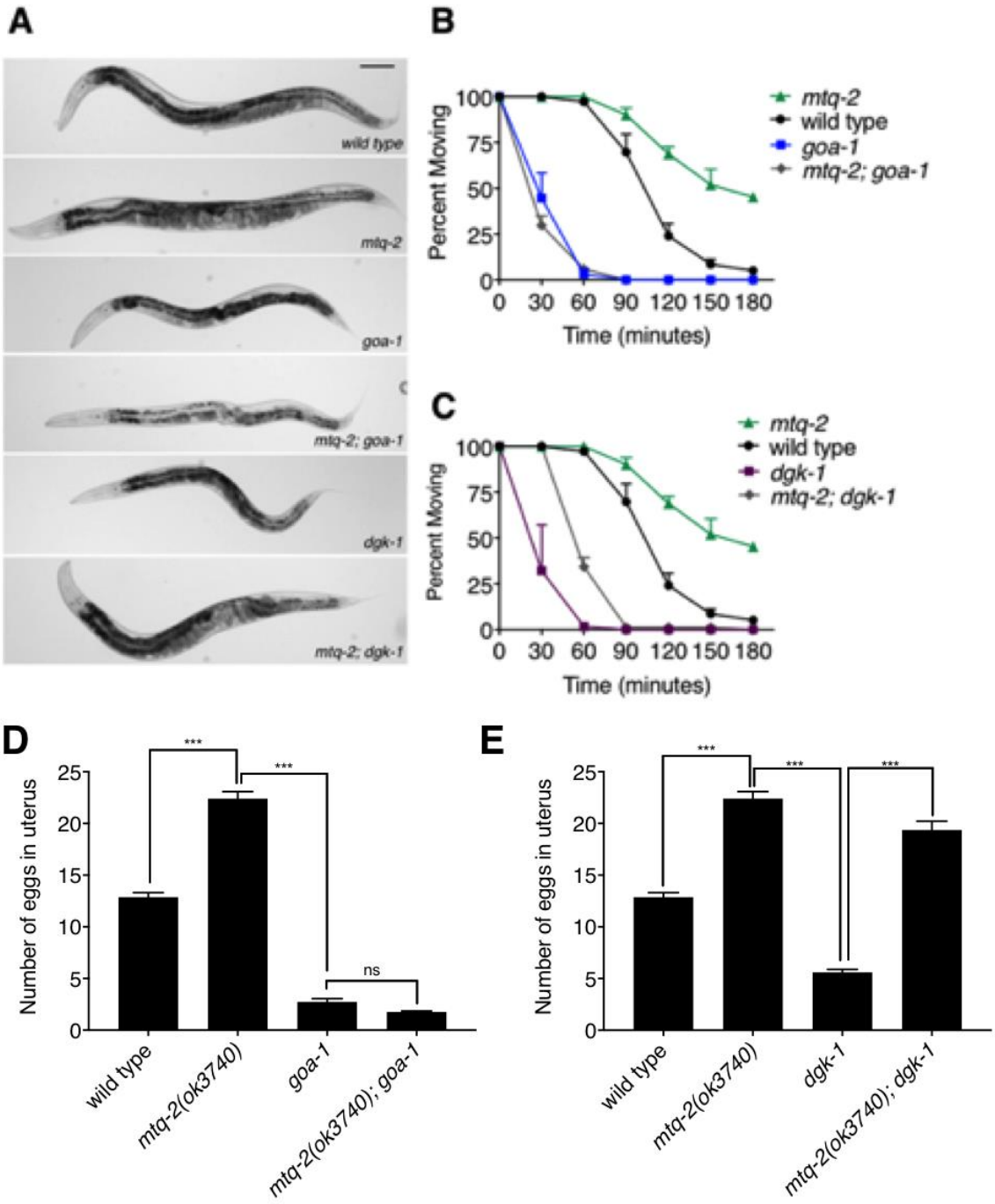

Figure 3.6. *mtq*-2 may regulate neurotransmitter release and egg laying via *goa-1* (Gαo)

Figure 3.6. *mtq*-2 may regulate neurotransmitter release and egg laying via *goa-1* (Gαo). **A.** Micrographs comparing visible phenotypes of wild-type, *mtq-2(ok3740)*, *goa-1*, *mtq-2; goa-1*, *dgk-1*, and *mtq-2; dgk-1*. Scale bar is 100 μm. Note that *mtq-2* mutants are bloated with eggs (in addition to being aldicarb resistant and having lower amplitude body bends). In contrast to *goa-1* mutants do not retain eggs. Double mutants of *mtq-2; goa-1* appear as the *goa-1* single mutant. **B-C.** Shown here is the time course to paralysis in the presence of 1-mM aldicarb. Mean percent of responsive animals are plotted. Error bars indicate s.e.m. Trials for all strains were performed at least four times. **B.** Mutants for *mtq-2* are moderately resistant to the paralytic effects of aldicarb while *goa-1* mutants are strongly hypersensitive. Double mutants of *mtq-2; goa-1* display a time course to paralysis indistinguishable from the *goa-1* single mutants. **C.** Mutants for *mtq-2* are moderately resistant to the paralytic effects of aldicarb while *dgk-1* mutants are strongly hypersensitive. Double mutants of *mtq-2; dgk-1* display a time course to paralysis intermediary to *mtq-2* and *dgk-1* single mutants. **D-E.** Shown is the mean number of eggs retained in the uterus of staged day one animals (L4 +- 24). Error bars indicate s.e.m. At least thrity animals per strain were scored. **D.** *mtq-2* mutants retain eggs while *goa-1* mutants constitutively lay eggs. Double mutants of *mtq-2; goa-1* appear as the *goa-1* single mutant. **E.** Double mutants of *mtq-2; dgk-1* appear to have a phenotype intermediary to the *mtq-2* and *dgk-1* single mutants.

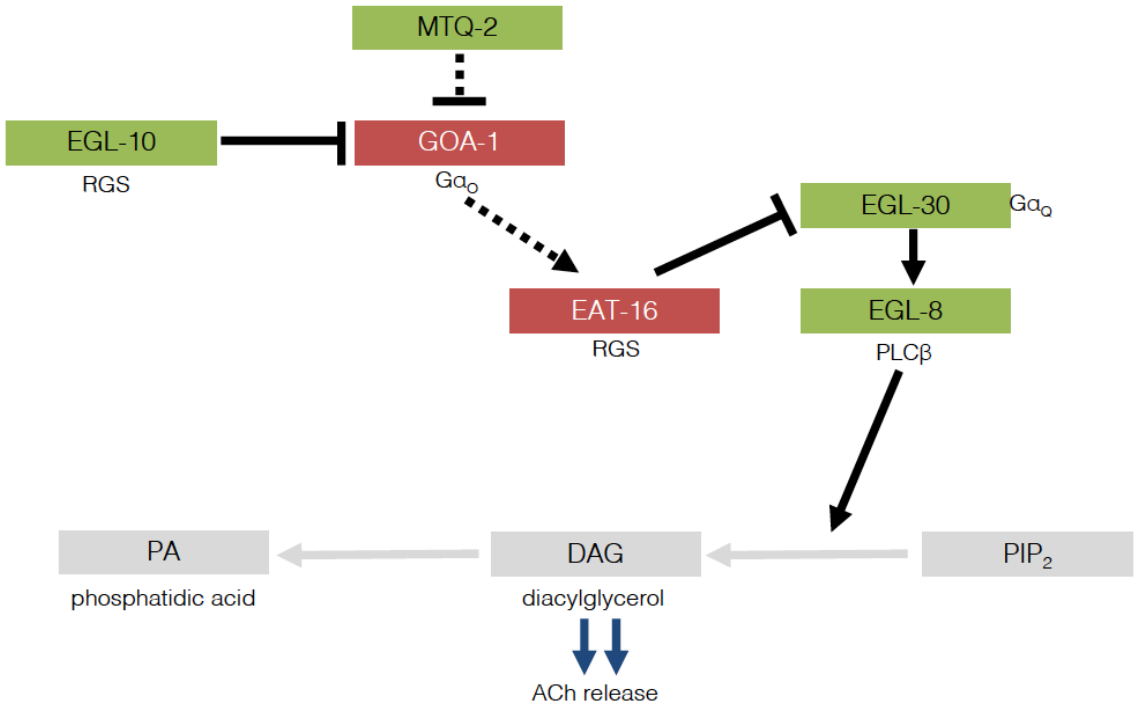

Figure 3.7. Proposed model for regulation of synaptic vesicle release by *mtq*-2. Green boxes indicate *C. elegans* proteins known to stimulate ACh release. Red boxes indicate proteins known to inhibit ACh release. Gray boxes show second messenger molecules. Solid lines and arrows indicate direct genetic interactions while dashed lines indicate the possibility of intermediates. Double blue arrows indicate the presence of downstream intermediates. Our data suggest MTQ-2 acts upstream of GOA-1 to inhibit the function of GOA-1. Research from other laboratories has determined all other downstream effectors of GOA-1. Adapted from (Bastiani & Mendel, 2006).

CHAPTER FOUR

CONCLUDING REMARKS AND FUTURE DIRECTIONS

CONCLUDING REMARKS AND FUTURE DIRECTIONS

In this thesis I functionally characterized all human 21st chromosome orthologs in *C. elegans*. The promise of this work lies in its potential. Candidate genes I identified are of immediate interest to Down syndrome research efforts and may also be useful for understanding phenotypes associated with genomic copy-number variation (CNV). Of the ten genes, I identified as required for normal behaviors in worm, six are inviable in mouse. This finding inadvertently highlights the importance of relying on multiple animal models to elucidate the *in vivo* function of genes.

Implications of Functional Screen of HSA21 Orthologs

Positive genes that came out of my screen can be grouped into the following categories: 1) genes with previously established links to the nervous system, 2) genes well-studied outside of the context of the nervous system, and 3) poorly characterized or novel genes weakly linked to the nervous system if at all.

As expected, functional screening identified genes with established links to nervous system function in both worm and mammal. Genes in this category include: *eva-1* (*EVA1C*), *ncam-1* (*NCAM2*), and *unc-26* (*SYNJ*). Two of these genes, *eva-1* (*EVA1C*) and *ncam-1* (*NCAM2*), are involved in axon guidance in worm, while *unc-26* (*SYNJ*) mediates synaptic vesicle release. In mouse, *EVA1C*, *NCAM2*, and *SYNJ* function similarly to their worm orthologs. *EVA1C*, a Slit receptor, has been proposed to mediate mammalian axon guidance on the basis of its axonal expression pattern in the developing brain, and *NCAM2*, a neural

cell adhesion molecule, has been shown to stimulate neurite branching in mouse cortical neurons (James, Foster, Key, & Beverdam, 2013; Sheng, Leshchyns'ka, & Sytnyk, 2015). Finally, *SYNJ1*, synaptojanin, mediates synaptic vesicle recycling in mammal as it does in worm (Cremona et al., 1999; Harris et al., 2000). Of note, both *eva-1* and *unc-26* were both identified and initially characterized in *C. elegans* (Brenner, 1974; Fujisawa et al., 2007). These findings support the use of *C. elegans* to guide research in mammals and demonstrate the predictive value of functions ascribed to *C. elegans* genes.

I identified a novel neuronal phenotype for, *rnt-1 (RUNX1)*, a transcription factor well-studied in worm for its role in general development, including cell proliferation and development of the male tail (Hajduskova et al., 2009; Nimmo et al., 2005). More recent research in mammal has revealed that *RUNX1* plays an essential role in the developing nervous system (Wang & Stifani, 2017). The radial dispersion assay I used to detect locomotor phenotypes of *rnt-1* mutants is quick, straightforward, and ideal for a forward genetic screen in the future.

My screen also unearthed three genes that are poorly characterized with respect to the nervous system: *dnsn-1 (DONSON), pdxk-1 (PDXK),* and *mtq-2 (N6AMT1)*. I further characterized *mtq-2*, which I will discuss in a later section; however, all three of genes deserve more detailed analysis to determine their precise function and mechanism of action.

I found that *dnsn-1* mutants displayed an extended dwelling phenotype, suggestive of deficits to sensory and/ or motor neuron function or development. Additionally, I found that a transcriptional reporter of *dnsn-1* expressed only

during the egg stage, further hinting at a possible role in development. The mammalian ortholog of *dnsn-1*, *DONSON*, was only recently described. Through whole-exome sequencing of patients with microcephalic dwarfism, Reynolds found that mutations in *DONSON* underlay the disorder (Reynolds et al., 2017). Prior to the Reynolds study, *Drosophila* researchers had already characterized the fly ortholog of *DONSON*, humpty-dumpty (*hd*). *Hd* mutants (as their name divertingly suggests) have very thin egg shells, a phenotype characteristic of genes involved in DNA replication (Bandura et al., 2005). In fly, Bandura confirmed that *hd* was essential for ovarian DNA replication and cell proliferation during brain development (Bandura et al., 2005). Informed by the fly phenotypes, Reynolds found that human *DONSON* also functioned in DNA replication as a novel replication fork protein (Reynolds et al., 2017).

In my screen of HSA21 orthologs, I also showed that *pdxk-1 (PDXK)* mutants were defective in several behaviors, including appropriate response to the acetylcholinesterase inhibitor aldicarb. The biochemical function of *PDXK* has been worked out: *PDXK* phosphorylates vitamin B6, converting it to PLP (pyridoxal-5'-phosphate), a key cofactor in the metabolism of hundreds of enzymatic reactions, including synthesis of neurotransmitters (Cao et al., 2006; Shetty & Gaitonde, 1980). The behavioral role of *PDXK* in mammal is less clear, probably, in part, because loss of *Pdxk* is pre-weaning lethal in mouse (Blake et al., 2017). In worm, I found that RNAi knockdown of *pdxk-1* caused aldicarb resistance—a compelling result; however, there were no characterized loss-of-function or deletion alleles. I identified three conserved residues on *pdxk-1* corresponding to uncharacterized point mutations in alleles from the Million

Mutation Project (MMP) (**Figure 4.1**). The Million Mutation Project is a collaborative effort in which *C. elegans* strains bearing many mutations are generated and mapped with whole-genome sequencing. I examined the phenotypes of all three MMP alleles. The strain harboring a M271I mutation (VC20794) appeared severely sick and I was unable to outcross it despite repeated attempts. I did not pursue study of this strain further. The second strain, VC40336, harboring a T155I mutation, exhibited a wild-type aldicarb response. The third strain, VC40866, containing an E148K mutation, displayed aldicarb resistance and behavioral deficits, which were weakly rescued with extrachromosomal expression of wild-type *pdxk-1* (**Figure 2.6**). I had difficulty generating viable animals carrying an extrachromosomal array of *pdxk-1*. I was only able to create strains carrying *pdxk-1* as an array by injecting a very low concentration of DNA (1 ng/ μl), which, possibly, suggests dose-sensitivity of the gene. As the human ortholog of *pdxk-1* is also predicted to be dose-sensitive (Antonarakis, 2016), further examination of this gene is merited. Ideally, CRISPR-based methods could be used to generate a null allele, which—if inviable—could be maintained as a heterozygote. Finally, the GABA neuron specific expression pattern I observed with a transcriptional reporter of *pdxk-1* was unexpected and, frankly, odd. *C. elegans* mutants defective in GABA signaling typically display several distinctive phenotypes (i.e. altered defecation cycle, abnormal response to mechanical stimuli), which I did not observe in *pdxk-1* mutants (McIntire, Jorgensen, Kaplan, & Horvitz, 1993). Due to the compactness of the worm's nervous system, deficiencies in GABA signaling can also cause an altered response to aldicarb (Vashlishan et al., 2008); yet, I would encourage anyone

wishing to pursue this gene to attempt to tag the protein with CRISPR-based techniques to confirm the expression pattern.

In addition to the novel genes I identified in my screen, there are additional orthologs that are inviable in worm and were not examined. Some of these genes are already known to express in the nervous system, including *atp-3* (*ATP5O*) and *ubc-14* (*UBE2G2*) **(Table 4.1**). It would be interesting to characterize these genes following mosaic knockdown in neurons, which could suggest a mechanism of action otherwise obscured.

Finally, overexpression techniques can be used to further characterize HSA21 orthologs in worm. I examined overexpression phenotypes of two candidate genes: *pdxk-1* and *mtq-2*. Overexpression of *mtq-2* resulted in pronounced sterility, which I will discuss in the next section. As previously noted, I had difficulty generating animals bearing *pdxk-1* as an extrachromosomal array and successfully created transformants only by injecting a very low concentration (1ng/ μl) of DNA. Among these transformants overexpressing *pdxk-1* at low concentration, I did not observe any obvious phenotypes. When dealing with extrachromosomal arrays, the following truism in biology especially applies: positive results are informative; negative results are far less telling. In general, *C. elegans* appear to be fairly tolerant to physiologically relevant increases in the dosage of wild-type genes (Hodgkin, 2005). Multi-copy array overexpression of certain genes, however, is sometimes not possible; and viable strains can only be created with single-copy integration (Hodgkin, 2005). I favor the use creating integrated, single-copy transgenic strains to examine overexpression phenotypes of both *pdxk-1* and *mtq-2* in the future. Regrettably,

creating integrated, single-copy transgenic strains is not a high-throughput technique; yet, the initial investment of labor can instill greater confidence in any results garnered from such studies.

Finally, it is my sincere hope that Down syndrome researchers employing mouse models can further examine the candidate genes I identified. There are two gold standard experiments that could be performed. First, transgenic mice overexpressing a single candidate gene could be generated and phenotypically characterized. Positive results from this experiment would indicate if overexpression of a candidate gene at physiologically relevant levels is sufficient to confer a phenotype. In mouse, transgenic overexpression of a wild-type HSA21-related gene can cause abnormal neurological phenotypes. For instance, overexpression of synaptojanin, *Synj1*, causes subtle spatial memory defects, while overexpression of *Dyrk1a* causes severe spatial memory defects, learning difficulties and altered synaptic plasticity (Ahn et al., 2006; Altafaj et al., 2001; Kleschevnikov et al., 2004; Voronov et al., 2008). Second, a candidate gene could be restored to disomic levels within a Down syndrome mouse model. Restoring a single gene to disomic levels on a Down syndrome mouse background can most readily be executed by a simple genetic cross; however, this does require haplosufficiency of the gene of interest. Results from these experiments can indicate the function of a given gene within the context of Down syndrome.

Future Directions for MTQ-2

In further characterizing a novel candidate gene that emerged from my screen, I found that *mtq-2* is required for normal nervous system function,

normal excitatory neurotransmission, and that it functions, specifically, in cholinergic neurons to mediate its effects. Initial genetic epistasis analysis suggests *mtq-2* may function upstream of or in conjunction with at least one Gα signaling protein, Gαo. These results are intriguing, and, I believe, sufficient to justify closer examination of *mtq-2* in mammals. Though mice homozygous null for *mtq-2* (*N6Amt1*) die very in embryonic development, the consequences of overexpression of this gene in mice are unknown (P. Liu et al., 2010).

There are still many unanswered questions regarding the mechanism of action of *mtq-2*. Critically, biochemical experiments are needed to determine the following: 1) What does MTQ-2 bind in worm? 2) Is the protein methylome altered in an *mtq-2* background, or more feasibly 3) Is *goa-1* (Gαo) methylated at glutamine? If so, 4) is *mtq-2* required for this modification? Positive results from the latter two questions would suggest a remarkable level of fine tuning of a key synaptic protein and suggest a functional role for non-histone glutamine methylation, a scarcely described post-translational modification.

If the aforementioned biochemical experiments do not support an interaction between *goa-1* (Gαo) and *mtq-2*, then alternate possibilities must be considered. In both yeast and mouse, MTQ-2 has been shown to partner with an adaptor subunit, TRM112. Though the gene (*C04H5.1*) encoding the adaptor subunit is well-conserved in worm, it has no known deletion or loss-of-function alleles and we did not pursue it. In yeast, both Liger and Figaro found that TRM112 functioned as a hub protein within a small network of genes including two tRNA methyltransferases, the protein methyltransferase, *mtq-2*, and an rRNA methyltransferase (**Figure 4.2**) (Figaro et al., 2012; Liger et al., 2011). Figaro

hypothesized that disrupting the levels of any of the catalytic subunits could perturb the function of the others by disrupting the normal stoichiometry of subunits in the methyltransferase complex. In worm, all of the genes in this small network are conserved but uncharacterized. Curiously, of those genes with known expression patterns, *C35D10.12 (TRM9)* and *Y71F9AL.1 (TRM11)* appear quite similar to the expression pattern I found for *mtq-2*. This raises the possibility that they may work together for some common purpose in the nervous system (**Figure 4.2**). Again, biochemical assays would help expedite an understanding of the interacting partners of *mtq-2*.

Finally, *mtq-2* may have an unappreciated role in development. Regarding nervous system development, I examined the localization, distribution, and relative fluorescence intensity of cholinergic synapses in the DA9 neuron and did not find major differences between wild-type animals and *mtq-2* mutants (**Figure 3.5**). However, both *mtq-2* mutants and strains overexpressing *mtq-2* exhibited several other phenotypes suggestive of developmental deficits. First, I consistently noted that *mtq-2* mutants were slightly delayed (~8 hours) in reaching a distinctive larval stage (L4). The delay in reaching the L4 stage may be due to general growth deficits or to eggs being laid at a premature stage. Future experiments dissecting the stage of eggs *in utero* in *mtq-2* mutants could support or controvert the latter possibility. Second, I found that overexpression of *mtq-2* in a wild-type background under its endogenous promoter caused pronounced sterility (**Figure 4.3),** which was possibly dose-dependent. Like growth delay, sterility can be caused by many factors, and it is not clear from my work why overexpression of *mtq-2* resulted in sterility. Interestingly, reduction in function

of one of the candidate interacting partners of *mtq-2* that I identified, *goa-1* (Gαo), is also associated with developmental defects, including sterility (Miller & Rand, 2000).

Final Remarks

For decades, functional analysis of 21st chromosome genes has been a priority within the field of Down syndrome research. Ignorance of the *in vivo* function of genes on the 21st chromosome, hobbles efforts to fully understand the genetic basis of the disorder. The research I conducted in this thesis is a step forward. Importantly, over half of the genes I identified in my systematic screen of 21st chromosome orthologs are inviable in mice, which may explain why some of them had been overlooked. To truly appreciate Down syndrome, it is imperative for researchers to rely on multiple approaches and multiple animal models. Discoveries in invertebrates may not always translate to mammals, but the return is immense when they do.

Human Gene	Worm Gene	Function	Expression
ATP5O	*atp-3*	ATP synthase subunit	nervous system[1, 2]
CCT8	*cct-8*	chaperonin subunit	-
CHAF1B	*chaf-2*	chromatin assembly factor	-
HSPA13	*stc-1*	ATPase	intestine[1, 2]
MRPL39	*mrpl-39*	mitochondrial ribosomal protein	-
MRPS6	*mrps-6*	mitochondrial ribosomal protein	-
UBE2G2	*ubc-14*	ubiquitin conjugating enzyme	nervous system[1, 2]
WDR4	*wdr-4*	WD Repeat protein	-

Table 4.1. Well-conserved orthologs of HSA21 genes inviable in *C. elegans*
List of human genes, corresponding worm gene, function of gene in worm, and in which tissue the gene expresses (if known). – indicates unknown expression pattern. Data for expression pattern was gathered from WormBase (Lee et al., 2018). Specific citations are as follows: [1](McKay et al., 2003) and [2](Hunt-Newbury et al., 2007).

Figure 4.1. Alignment of *PDXK* and location of missense mutations
Human and *C. elegans PDXK* were aligned with Clustal Omega. Shading indicates residues affected by mutations in the Million Mutation Project (MMP). Gray shading indicates missense mutations in residues not conserved. Strains harboring mutations affecting residues shaded in blue were screened (VC20794, M271I and VC40336, T155I). Strain (VC40866) harboring E148K mutation red was screened and found to have behavioral and synaptic defects.

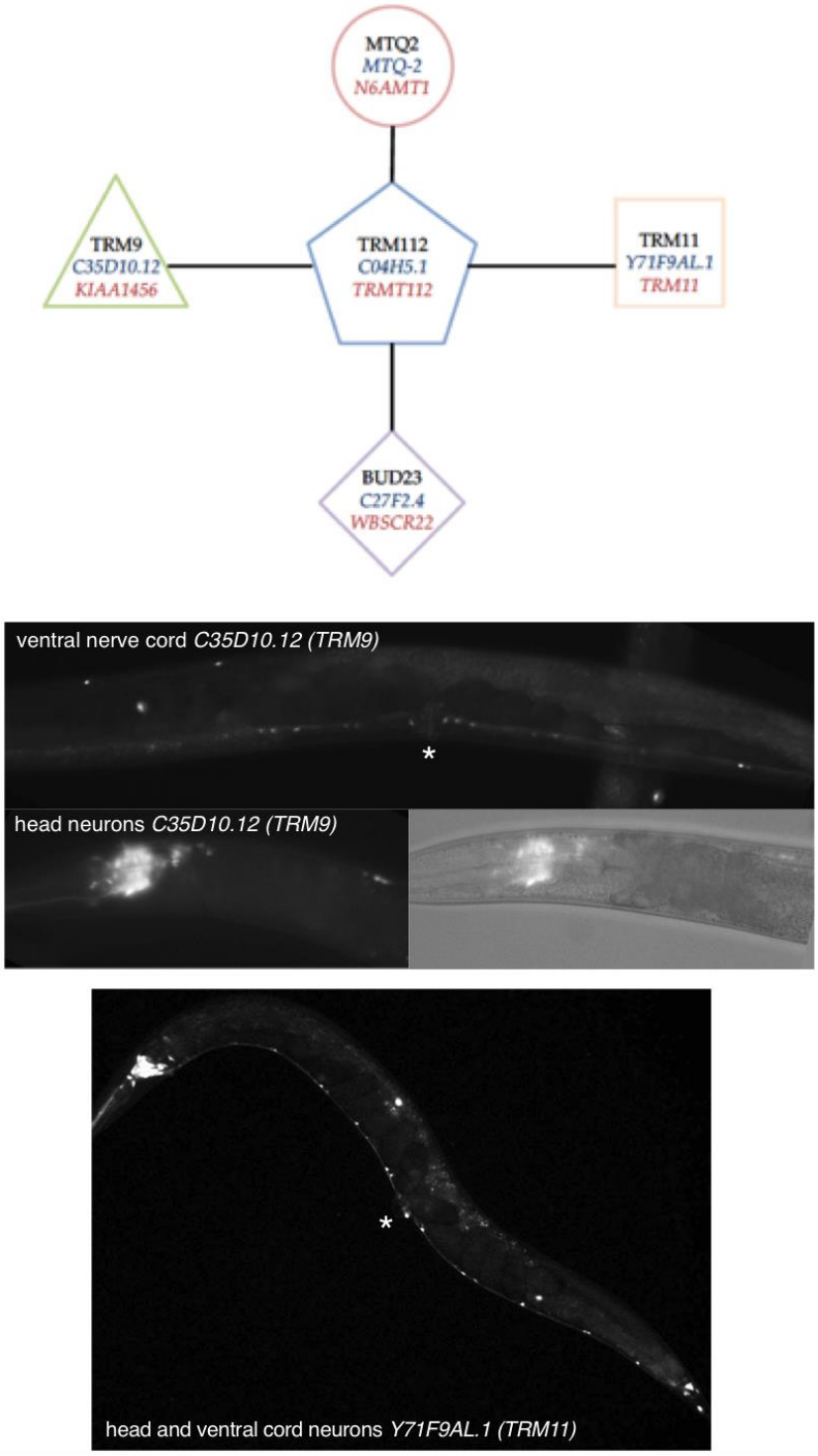

Figure 4.2. Partners and predicted partners of hub protein *TRM112*

Figure 4.2. Partners and predicted partners of hub protein *TRM112*
A. Schematic illustrating the four catalytic subunits known to associate with the hub protein, TRM112 in yeast. Model derived from (Figaro et al., 2012; Liger et al., 2011). Orthology was determined with InParanoid. Black text refers to the gene name *S. cerevisiae*; blue, *C. elegans*; and red, *H. sapiens*. **B.** Images of transcriptional reporters of *C.elegans* genes predicted, by homology, to participate in network. Asterisk indicates location of vulva. Images for *C35D10.12 (TRM9)* and *Y71F9AL.1 (TRM11)* were taken from the *C.elegans* Promoter Expression Database. For images of *mtq-2* see (Nordquist et al., 2018).

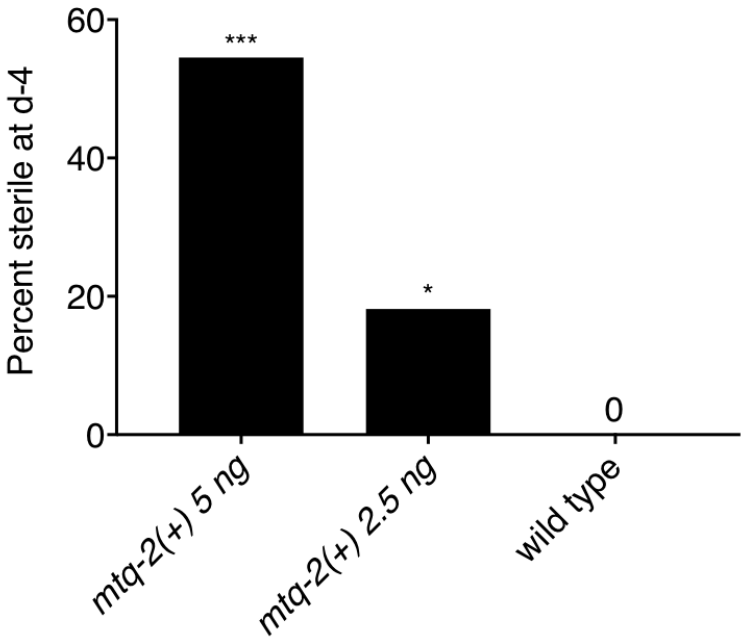

Figure 4.3. Overexpression of *mtq-2* causes sterility
Shown here is the mean percent sterile at day four of adulthood. At least 20 animals per strain tested. Significance (*$p < 0.05$, ***$p < 0.001$) determined with Fischer's exact test. 0 indicates no sterility.

APPENDICES

APPENDIX A. LIST OF HSA21 ORTHOLOGS SELECTED FOR STUDY

#Human Gene	Human Uniprot	Human Gene	Mouse Gene	DSCR	Ts65Dn	Worm Uniprot	Worm Gene	#Worm Gene
1	P78563	ADARB1	Adarb1			Q22618	*adr-2*	1
2	P48047	**ATP5O**	**Atp5o**		✓	**Q7JNG1**	***atp-3***	2
3	Q9Y2C3	B3GALT5	B3galt5	✓	✓	Q8MQG4	*B0024.15*	3
4	P35520	CBS	Cbs			Q93244	*cysl-1*	4
	P35520	CBS	Cbs			O45679	*cysl-2*	5
	P35520	CBS	Cbs			O01592	*cysl-3*	6
	P35520	CBS	Cbs			O16284	*cysl-4*	7
5	P50990	**CCT8**	**Cct8**		✓	**Q9N358**	***cct-8***	8
6	Q13112	**CHAF1B**	**Chaf1b**	✓	✓	**Q95XL8**	***chaf-2***	9
7	Q96NY7	CLIC6	Clic6		✓	G5ED64	*D1086.9*	10
	Q96NY7	CLIC6	Clic6		✓	Q564Q6	*H39E23.3*	11
8	P39060	COL18A1	Col18a1			G5EGA4	*cle-1*	12
9	Q14689	DIP2A	Dip2a			Q1RS87	*dip-2*	13
10	Q9NYP3	**DONSON**	**Donson**		✓	**H2KYF1**	***dnsn-1***	14
11	Q9Y3R5	DOPEY2	Dopey2	✓	✓	Q9XW10	*pad-1*	15
12	O60469	DSCAM	Dscam	✓	✓	Q9TXI8	*igcm-1*	16
13	Q13627	DYRK1A	Dyrk1a	✓	✓	Q8WQL7	*mbk-1*	17
14	P58658	**EVA1C**	**Eva1c**		✓	**Q9XU98**	***eva-1***	18
15	P22102	**GART**	**Gart**		✓	**Q20143**	***F38B6.4***	19
16	P48723	**HSPA13**	**Hspa13**			**Q20752**	***stc-1***	20
17	P05107	ITGB2	Itgb2			Q27874	*pat-3*	21
18	Q15811	ITSN1	Itsn1		✓	Q9U2T9	*itsn-1*	22

APPENDIX A

19	P57087	JAM2	Jam2		✓	A8WFH3	*zig-10*	23
20	P48051	KCNJ6	Kcnj6	✓	✓	D3YT08	*irk-2*	24
21	O94822	**LTN1**	**Ltn1**		✓	**Q65XX2**	***Y54E10A.11***	25
22	Q9NYK5	**MRPL39**	**Mrpl39**		✓	**Q4W5T0**	***mrpl-39***	26
23	P82932	**MRPS6**	**Mrps6**		✓	**O61791**	***mrps-6***	27
24	Q9Y5N5	**N6AMT1**	**N6amt1**		✓	**O16582**	***mtq-2***	28
25	O15394	NCAM2	Ncam2			Q8MQ86	*ncam-1*	29
26	Q9Y5B6	PAXBP1	Paxbp1		✓	G5ECH1	*F43G9.12*	30
27	P57721	PCBP3	Pcbp3			Q95Y67	*pes-4*	31
28	O00764	**PDXK**	**Pdxk**			**O01824**	***pdxk-1***	32
29	P17858	PFKL	Pfkl			Q9TZL8	*pfk-1.1*	33
30	Q9Y2G5	**POFUT2**	**Pofut2**			**Q8WR51**	***pad-2***	34
31	Q86YR6	POTED	Poteg			G5EFS1	*ikb-1*	35
32	P53805	RCAN1	Rcan1		✓	P53806	*rcan-1*	36
33	P56182	RRP1	Rrp1			Q18674	*C47E12.7*	37
34	Q14684	RRP1B	Rrp1b			Q18674	*C47E12.7*	dup.
35	Q01196	RUNX1	Runx1		✓	G5EFQ5	*rnt-1*	38
36	O95104	SCAF4	Scaf4		✓	O01864	*nrd-1*	39
37	Q9NVD3	**SETD4**	**Setd4**	✓	✓	**Q9BPP1**	***set-29***	40
38	P55822	**SH3BGR**	**Sh3bgr**	✓	✓	**K8ES47**	***Y105E8A.1***	41
39	Q14190	SIM2	Sim2	✓	✓	P90953	*hlh-34*	42
40	P00441	SOD1	Sod1		✓		*sod-1*	43
	P00441	SOD1	Sod1		✓		*sod-5*	44
41	P18583	**SON**	**Son**		✓	**Q9TYS1**	***D1037.1***	45
42	O43426	SYNJ1	Synj1		✓	G5ECL2	*unc-26*	46
43	P56557	TMEM50B	Tmem50b		✓	Q9N2X7	*Y74C10AL.2*	47
44	P48553	**TRAPPC10**	**Trappc10**			**B1V899**	***trpp-10***	48
45	P60604	**UBE2G2**	**Ube2g2**			**Q9U1Q1**	***ubc-14***	49

46	Q9UHP3	USP25	Usp25			Q09931	*K02C4.3*	50
47	P57081	**WDR4**	**Wdr4**			**Q23232**	***wdr-4***	51

APPENDIX A. LIST OF HSA21 ORTHOLOGS SELECTED FOR STUDY

List of 47 human genes on 21st chromosome with one or more *C. elegans* orthologs predicted by the InParanoid algorithm. Because the 47 human genes are represented by 51 genes in *C. elegans*, several human genes are matched to multiple worm genes. For instance, the human gene CBS is matched to worm genes: *cysl-1*, *cysl-2*, *cysl-3*, and *cysl-4*. In only one case two paralogous human genes (RRP1 and RRP1B) are matched to a single worm gene in (*C47E12.7*), indicated by the text "dup." for duplicate. Of the 47 human genes, 19 are matched one-to-one with worm gene (bold), 25 are matched many-to-one with (25) worm genes, and 3 are matched one-to-many (8) worm genes. In total, the 47 human genes are represented by 51 unique orthologs in *C. elegans*. To demonstrate this relation, unique human and worm genes are numbered on left and right sides. Columns list corresponding Uniprot ID for human gene, mouse orthologous gene, whether the gene has been reported to lie within the so-called Down syndrome critical region (DSCR) ((X. Jiang et al., 2015), whether the corresponding mouse gene is overexpressed in the common mouse model of Down syndrome Ts65Dn ((Olson et al., 2004), the worm Uniprot identification code, and worm gene name.

APPENDIX B. COVERAGE AND REPLICATION OF RNAI SCREEN OF HSA21 ORTHOLOGS SELECTED FOR SCREEN

Human Gene	Worm Gene	RNAi Probe	Not Viable	Not Viable Replicated	WT	WT Replicated	Conflict
ADARB1	*adr-2*	III-3J14			✓	✓	
ATP5O	***atp-3***	**I-2O16**	✓	✓			
B3GALT5	*B0024.15*	V-7K15			✓		
CBS	*cysl-1*	X-4L22			✓	✓	
CBS	*cysl-2*	II-9M07			✓	✓	
CBS	*cysl-3*	V-3C18			✓	✓	
CBS	*cysl-4*	V-2A09			✓		
CCT8	***cct-8***	**IV-8P07**	✓	✓			
CHAF1B	***chaf-2***	**I-8O03**			✓		✓
CLIC6	*D1086.9*	-					
CLIC6	*H39E23.3*	-					
COL18A1	*cle-1*	I-4E22			✓	✓	
DIP2A	*dip-2*	I-2M19	✓	✓			
DONSON	***dnsn-1***	**II-1O15**	✓	✓			
DOPEY2	*pad-1*	I-6F05	✓	✓			
DSCAM	*igcm-1*	X-8D03			✓		
DYRK1A	*mbk-1*	X-6J16			✓	✓	
EVA1C	***eva-1***	**I-7I22**			✓	✓	
GART	***F38B6.4***	**X-3F13**			✓	✓	
HSPA13	***stc-1***	**II-6A08**	✓	✓			
ITGB2	*pat-3*	III-1P02	✓	✓			

APPENDIX B.

ITSN1	*itsn-1*	IV-8I23			✓	✓	
JAM2	*zig-10*	II-5A05			✓	✓	
KCNJ6	*irk-2*	X-1I21			✓		✓
LTN1	***Y54E10A.11***	**-**					
MRPL39	***mrpl-39***	**V-1L20**	✓	✓			
MRPS6	***mrps-6***	**I-1H04**	✓	✓			
N6AMT1	***mtq-2***	**II-2M20**			✓	✓	
NCAM2	*ncam-1*	X-1I17			✓	✓	
PAXBP1	*F43G9.12*	I-4C12	✓	✓			
PCBP3	*pes-4*	III-8I07	✓	✓			
PDXK	***pdxk-1***	**I-2K05**			✓	✓	
PFKL	*pfk-1.1*	X-2C11	✓	✓			
POFUT2	***pad-2***	**III-8G24**			✓	✓	
POTED	*ikb-1*	I-4H10			✓	✓	
RCAN1	*rcan-1*	III-3A09			✓	✓	
RRP1	*C47E12.7*	IV-5O03	✓	✓			
RRP1B	*C47E12.7*	dup.	dup.	dup.			
RUNX1	*rnt-1*	I-2L23			✓	✓	
SCAF4	*nrd-1*	I-2A21	✓	✓			
SETD4	***set-29***	**-**					
SH3BGR	***Y105E8A.1***	**I-9O01**			✓		
SIM2	*hlh-34*	V-9C07			✓	✓	
SOD1	*sod-1*	II-5G04			✓		
SOD1	*sod-5*	II-3F14			✓	✓	
SON	***D1037.1***	**I-1F05**			✓	✓	
SYNJ1	*unc-26*	IV-7E19			✓	✓	
TMEM50B	*Y74C10AL.2*	-					

TRAPPC10	***trpp-10***	**I-9P01**			✓	✓	
UBE2G2	***ubc-14***	**-**					
USP25	*K02C4.3*	II-5P18			✓	✓	
WDR4	***wdr-4***	**III-3F21**			✓	✓	

APPENDIX B. COVERAGE AND REPLICATION OF RNAI SCREEN OF HSA21 ORTHOLOGS SELECTED FOR SCREEN

Display of the degree of coverage and replication for our RNAi analysis. We list 46 RNAi probes from the Ahringer library used for phenotypic testing. Only 6 HSA21 orthologs did not have a corresponding RNAi clone. Checkmarks indicate phenotypes we found that may preclude study of these genes in loss-of-function mutations including embryonic lethal (Emb), sterile (Ste), severe growth defect (Gro), and larval arrest (Lva). ((Ceron et al., 2007; Cui et al., 2008; A. G. Fraser et al., 2000; Gottschalk et al., 2005; Jones, Crowe, Stevens, & Candido, 2002; Kamath et al., 2003; Maeda et al., 2001; Nakano et al., 2011; Pujol et al., 2001; Rual et al., 2004; Simmer et al., 2003; Sönnichsen et al., 2005).

APPENDIX C. COVERAGE AND REPLICATION OF MUTANT SCREEN OF HSA21 ORTHOLOGS SELECTED FOR STUDY

Human Gene	Worm Gene	Strain	Tested or Not Viable	Pheno.	1st Report	Pheno. Rep.	Conflict
ADARB1	*adr-2*	BB3	✓				
ATP5O	***atp-3***	-	✓				
B3GALT5	*B0024.15*	FX6706	✓				
CBS	*cysl-1*	RB899	✓				
CBS	*cysl-2*	RB2535	✓	✓	✓		
CBS	*cysl-3*	-					
CBS	*cysl-4*	RB2436	✓				
CCT8	***cct-8***	-	✓				
CHAF1B	***chaf-2***	-	✓				
CLIC6	*D1086.9*	-					
CLIC6	*H39E23.3*	-					
COL18A1	*cle-1*	VC943	✓	✓	✓		
DIP2A	*dip-2*	RB979	✓				
DONSON	***dnsn-1***	FX3322	✓	✓	✓		
DOPEY2	*pad-1*	-	✓				
DSCAM	*igcm-1*	RB870	✓				
DYRK1A	*mbk-1*	EK228	✓				
EVA1C	***eva-1***	VC868	✓	✓	✓		
GART	***F38B6.4***	-					
HSPA13	***stc-1***	-	✓				
ITGB2	*pat-3*	-	✓				

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ITSN1	*itsn-1*	VC201	✓				
JAM2	*zig-10*	FX6327	✓				
KCNJ6	*irk-2*	-	✓				
LTN1	***Y54E10A.11***	-					
MRPL39	***mrpl-39***	-	✓				
MRPS6	***mrps-6***	-	✓				
N6AMT1	***mtq-2***	FX3565	✓	✓	✓		
NCAM2	*ncam-1*	VH860	✓	✓	✓		
PAXBP1	*F43G9.12*	-	✓				
PCBP3	*pes-4*	-	✓				
PDXK	***pdxk-1***	VC40866	✓	✓	✓		
PFKL	*pfk-1.1*	-	✓				
POFUT2	***pad-2***	FX1756	✓	✓	✓		
POTED	*ikb-1*	-					
RCAN1	*rcan-1*	FX2021	✓				
RRP1	*C47E12.7*	-	✓				
RRP1B	*C47E12.7*	-	dup.				
RUNX1	*rnt-1*	VC200	✓	✓	✓		
SCAF4	*nrd-1*	FX2657	✓				
SETD4	***set-29***	RB2097	✓				
SH3BGR	***Y105E8A.1***	FX2626	✓				
SIM2	*hlh-34*	-					
SOD1	*sod-1*	FX776	✓				
SOD1	*sod-5*	GA503	✓				
SON	***D1037.1***	RB1489	✓				
SYNJ1	*unc-26*	DR97	✓	✓		✓	
TMEM50B	*Y74C10AL.2*	-					
TRAPPC10	***trpp-10***	-					

UBE2G2	***ubc-14***	-	✓				
USP25	*K02C4.3*	-	✓				
WDR4	***wdr-4***	-	✓				

APPENDIX C. COVERAGE AND REPLICATION OF MUTANT SCREEN OF HSA21 ORTHOLOGS SELECTED FOR STUDY

Display of the degree of coverage and replication of our mutant analysis. We list the 30 outcrossed, homozygous viable strains with predicted loss-of-function mutations that we used to examine neuronal phenotypes in this study. There were also 12 orthologs for which RNAi or mutant data suggest inviability when mutated. Only 9 orthologs were not accounted for by a mutant or evidence of inviability. Mutant phenotypes detected in our study are listed. Only one of these genes (*unc-26*) had previously described mutant phenotypes (Harris et al., 2000).

APPENDIX D. OTHER PUTATIVE HSA21 ORTHOLOGS NOT SELECTED FOR SCREEN

# Human gene	Human Uniprot	Human Gene	Worm Gene	# Worm gene
1	P45844	ABCG1	*wht-2*	1
	P45844	ABCG1	*wht-3*	2
	P45844	ABCG1	*wht-6*	3
	P45844	ABCG1	*wht-8*	4
	P45844	ABCG1	*wht-1*	5
	P45844	ABCG1	*wht-4*	6
	P45844	ABCG1	*wht-5*	7
	P45844	ABCG1	*wht-7*	8
2	Q9UHI8	ADAMTS1	*gon-1*	9
3	Q9UNA0	ADAMTS5	*gon-1*	
4	P05067	APP	*apl-1*	10
-	Q9Y2C3	B3GALT5	*bre-2*	11
	Q9Y2C3	B3GALT5	*C47F8.3*	12
	Q9Y2C3	B3GALT5	*C47F8.5*	13
	Q9Y2C3	B3GALT5	*C47F8.6*	14
	Q9Y2C3	B3GALT5	*C54C8.3*	15
	Q9Y2C3	B3GALT5	*E03H4.11*	16
	Q9Y2C3	B3GALT5	*F14B6.4*	17
	Q9Y2C3	B3GALT5	*F14B6.6*	18
	Q9Y2C3	B3GALT5	*T09E11.10*	19
	Q9Y2C3	B3GALT5	*T09F5.1*	20
	Q9Y2C3	B3GALT5	*T15D6.5*	21
5	Q9Y5Z0	BACE2	*asp-1*	22

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	Q9Y5Z0	BACE2	*asp-2*	23
	Q9Y5Z0	BACE2	*asp-5*	24
	Q9Y5Z0	BACE2	*asp-6*	25
	Q9Y5Z0	BACE2	*C11D2.2*	26
	Q9Y5Z0	BACE2	*C15C8.3*	27
	Q9Y5Z0	BACE2	*F21F8.2*	28
	Q9Y5Z0	BACE2	*F21F8.4*	29
	Q9Y5Z0	BACE2	*F28A12.4*	30
	Q9Y5Z0	BACE2	*F59D6.2*	31
	Q9Y5Z0	BACE2	*F59D6.3*	32
	Q9Y5Z0	BACE2	*K10C2.3*	33
	Q9Y5Z0	BACE2	*Y39B6A.22*	34
	Q9Y5Z0	BACE2	*Y39B6A.23*	35
	Q9Y5Z0	BACE2	*Y39B6A.24*	36
	Q9Y5Z0	BACE2	*ZK384.3*	37
	Q9Y5Z0	BACE2	*ZK384.6*	38
6	O43822	C21orf2	*F09G8.5*	39
7	Q9Y426	C2CD2	*R11G1.6*	40
8	P16152	CBR1	*dhs-31*	41
9	O75828	CBR3	*dhs-31*	
-	P35520	CBS	*cbs-1*	42
	P35520	CBS	*cbs-2*	43
	P35520	CBS	*F59A7.7*	44
-	Q96NY7	CLIC6	*exc-4*	45
	Q96NY7	CLIC6	*exl-1*	46
10	P12109	COL6A1	*C16E9.1*	47
	P12109	COL6A1	*C18H7.1*	48
	P12109	COL6A1	*cutl-23*	49

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11	P12110	COL6A2	*C16E9.1*	50
	P12110	COL6A2	*C18H7.1*	51
	P12110	COL6A2	*cutl-23*	52
12	P02489	CRYAA	*hsp-12.1*	53
	P02489	CRYAA	*hsp-12.2*	54
	P02489	CRYAA	*hsp-12.3*	55
	P02489	CRYAA	*hsp-12.6*	56
13	P11308	ERG	*ast-1*	57
	P11308	ERG	*ets-5*	58
14	P15036	ETS2	*ets-7*	59
	P15036	ETS2	*ets-9*	60
	P15036	ETS2	*F19F10.1*	61
15	P58499	FAM3B	*M70.4*	62
	P58499	FAM3B	*Y73B3A.3*	63
16	Q06546	GABPA	*ets-7*	64
	Q06546	GABPA	*F19F10.1*	65
17	P39086	GRIK1	*glr-3*	66
	P39086	GRIK1	*glr-4*	67
	P39086	GRIK1	*glr-5*	68
	P39086	GRIK1	*glr-1*	69
	P39086	GRIK1	*glr-2*	70
18	P50747	HLCS	*bpl-1*	71
19	Q99712	KCNJ15	*irk-3*	72
20	O60318	MCM3AP	*F20D12.2*	73
21	Q14149	MORC3	*morc-1*	74
-	Q9NYK5	MRPL39	*tars-1*	75
22	P20591	MX1	*dyn-1*	76
23	P20592	MX2	*dyn-1*	

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24	Q8TAK6	OLIG1	*hlh-16*	77
25	Q13516	OLIG2	*hlh-16*	
26	O95613	PCNT	*lfi-1*	78
-	P17858	PFKL	*pfk-1.2*	79
-	Q86YR6	POTED	*act-1*	80
	Q86YR6	POTED	*act-2*	81
	Q86YR6	POTED	*act-3*	82
	Q86YR6	POTED	*act-4*	83
	Q86YR6	POTED	*act-5*	84
27	P53801	PTTG1IP	*C37C3.12*	85
28	Q15269	PWP2	*F55F8.3*	86
29	P57052	RBM11	*Y37D8A.21*	87
30	P41440	SLC19A1	*folt-1*	88
	P41440	SLC19A1	*folt-2*	89
	P41440	SLC19A1	*folt-3*	90
31	P57057	SLC37A1	*T10C6.6*	91
	P57057	SLC37A1	*T11G6.2*	92
	P57057	SLC37A1	*T11G6.3*	93
	P57057	SLC37A1	*T11G6.4*	94
-	P00441	SOD1	*sod-4*	95
-	P18583	SON	*C04G2.8*	96
	P18583	SON	*C10G11.9*	97
	P18583	SON	*T27A3.4*	98
32	P55854	SUMO3	*smo-1*	99
33	Q13009	TIAM1	*cgef-2*	100
34	O94759	TRPM2	*ced-11*	101
35	P53804	TTC3	*C09E7.7*	102
	P53804	TTC3	*C09E7.8*	103

	P53804	TTC3	*C09E7.9*	104
	P53804	TTC3	*F27B3.5*	105
36	Q01081	U2AF1	*uaf-2*	106
37	P57075	UBASH3A	*F09C12.8*	107
	P57075	UBASH3A	*F55A11.11*	108
	P57075	UBASH3A	*T07F12.1*	109
-	P60604	UBE2G2	*ubc-7*	110
38	O60287	URB1	*T05H4.10*	111

APPENDIX D. OTHER PUTATIVE HSA21 ORTHOLOGS NOT SELECTED FOR SCREEN

List of human 21st chromosome genes with one or more ortholog predicted by OrthoList but not InParanoid. There are 38 unique human genes (indicated with a number) in this category. The remaining 8 unique human genes (indicated with a dash) have an InParanoid defined ortholog listed in APPENDIX B. Because the 46 human genes are represented by 111 genes in *C. elegans*, most human genes are matched to multiple worm genes. e.g. The human gene ABCG1 is matched to four paralogs: *wht-2*, *wht-3*, *wht-6*, and *wht-8*. In four cases, paralogous human genes are matched to the same worm gene. e.g. OLIG1 and OLIG2 are both matched to *hlh-16* in worm.

APPENDIX E. ALPHABETICAL LIST OF HSA21 GENES WITH NO ORTHOLOG

# Human Gene	Human Gene	# Human Gene	Human Gene	# Human Gene	Human Gene	# Human Gene	Human Gene
1	AGPAT3	33	IFNAR1	65	**KRTAP19-4**	97	PCP4
2	AIRE	34	IFNAR2	66	**KRTAP19-5**	98	PDE9A
3	ATP5J	35	IFNGR2	67	**KRTAP19-6**	99	PIGP
4	BACH1	36	IGSF5	68	**KRTAP19-7**	100	PKNOX1
5	BAGE3	37	IL10RB	69	**KRTAP19-8**	101	PLAC4
6	BAGE4	38	KCNE1	70	**KRTAP20-1**	102	PRDM15
7	BRWD1	39	KCNE2	71	**KRTAP20-2**	103	PRMT2
8	BTG3	40	**KRTAP10-1**	72	**KRTAP20-3**	104	PSMG1
9	C21orf140	41	**KRTAP10-10**	73	**KRTAP21-1**	105	RIPK4
10	C21orf33	42	**KRTAP10-11**	74	**KRTAP21-2**	106	RIPPLY3
11	C21orf58	43	**KRTAP10-12**	75	**KRTAP21-3**	107	RSPH1
12	C21orf59	44	**KRTAP10-2**	76	**KRTAP22-1**	108	RWDD2B
13	C21orf62	45	**KRTAP10-3**	77	**KRTAP22-2**	109	S100B
14	C21orf91	46	**KRTAP10-4**	78	**KRTAP23-1**	110	SAMSN1
15	CHODL	47	**KRTAP10-5**	79	**KRTAP24-1**	111	SIK1
16	CLDN14	48	**KRTAP10-6**	80	**KRTAP25-1**	112	SLC5A3
17	CLDN17	49	**KRTAP10-7**	81	**KRTAP26-1**	113	SMIM11
18	CLDN8	50	**KRTAP10-8**	82	**KRTAP27-1**	114	SPATC1L
19	CRYZL1	51	**KRTAP10-9**	83	**KRTAP6-1**	115	TCP10L
20	CSTB	52	**KRTAP11-1**	84	**KRTAP6-2**	116	TFF1
21	CXADR	53	**KRTAP12-1**	85	**KRTAP6-3**	117	TFF2
22	CYYR1	54	**KRTAP12-2**	86	**KRTAP7-1**	118	TFF3
23	DNAJC28	55	**KRTAP12-3**	87	**KRTAP8-1**	119	TMPRSS15

24	DNMT3L	56	**KRTAP12-4**	88	LCA5L	120	TMPRSS2
25	DSCR3	57	**KRTAP13-1**	89	LIPI	121	TMPRSS3
26	DSCR4	58	**KRTAP13-2**	90	LRRC3	122	TPTE
27	FAM207A	59	**KRTAP13-3**	91	LSS	123	TSPEAR
28	FTCD	60	**KRTAP13-4**	92	MAP3K7CL	124	UMODL1
29	HMGN1	61	**KRTAP15-1**	93	MIS18A	125	USP16
30	HSF2BP	62	**KRTAP19-1**	94	MRAP	126	WRB
31	HUNK	63	**KRTAP19-2**	95	NDUFV3	127	YBEY
32	ICOSLG	64	**KRTAP19-3**	96	NRIP1	128	ZBTB21

APPENDIX E. ALPHABETICAL LIST OF HSA21 GENES WITH NO ORTHOLOG

Alphabetical list of 128 human genes (HGNC name) on 21st chromosome with no predicted ortholog in *C. elegans* (determined by OrthoList). The 48 keratin genes are listed in bold. The remaining 80 genes are listed in regular font.

APPENDIX F. LIST OF OUTCROSSED MUTANT STRAINS

Strain	Gene	Allele	Chromosome	Outcrossed #	Parent Strain	Source
BB3	*adr-2*	*gv42*	III	x		CGC
CB211	*lev-1*	*e211*	IV	x		CGC
CZ18537	*zig-10*	*tm6127*	II	x		Y. Jin
DR97	*unc-26*	*e345*	IV	x		CGC
EK228	*mbk-1*	*pk1389*	X	x		CGC
JPS641	*pad-2*	*tm1756*	III	3x	FX1756	NBRP
JPS686	*rcan-1*	*tm2021*	III	2x	FX2021	NBRP
JPS730	*Y105E8A.1*	*tm2626*	I	1x	FX2626	NBRP
JPS731	*nrd-1*	*tm2657*	I	1x	FX2657	NBRP
JPS636	*dnsn-1*	*tm3322*	II	3x	FX3322	NBRP
JPS637	*mtq-2*	*tm3565*	II	3x	FX3565	NBRP
JPS684	*B0024.15*	*tm6706*	V	1x	FX6706	NBRP
FX776	*sod-1*	*tm776*	II	x		NBRP
GA503	*sod-5*	*tm1146*	II	x		NBRP
MT1072	*egl-4*	*n477*	IV	x		CGC
N2						CGC
JPS689	*ikb-1*	*nr2027*	I	3x	NS3026	CGC
PR678	*tax-4*	*p678*	III	x		CGC
PS2627	*dgk-1*	*sy428*	X	x		CGC
JPS693	*D1037.1*	*ok1746*	I	1x	RB1489	CGC
JPS642	*set-29*	*ok2772*	I	3x	RB2097	CGC
JPS980	*cysl-4*	*ok3359*	V	3x	RB2436	CGC
RB2535	*cysl-2*	*ok3516*	II	x		CGC
JPS687	*igcm-1*	*ok711*	X	2x	RB870	CGC
JPS639	*cysl-1*	*ok762*	X	2x	RB899	CGC

JPS685	*dip-2*	*ok885*	I	1x	RB979	CGC
JPS690	*rnt-1*	*ok351*	I	1x	VC200	CGC
JPS683	*itsn-1*	*ok268*	IV	2x	VC201	CGC
JPS694	*mtq-2*	*ok3740*	II	2x	VC3037	CGC
JPS966	*pdxk-1*	*gk855208*	I	3x	VC40866	CGC
VC868	*eva-1*	*ok1133*	I	x		CGC
JPS638	*cle-1*	*gk421*	I	1x	VC943	CGC
VH860	*ncam-1*	*hd49*	X	x		H. Hutter

APPENDIX F. LIST OF OUTCROSSED MUTANT STRAINS

List of all outcrossed strains used in study, genotype, number of times outcrossed, parent strain from which outcrossed strain was derived, and the source of the strain. A single x indicates that the strain has been outcrossed an indeterminate number of times by outside laboratories. CGC, *Caenorhabditis Genetics Center*. NBRP, National BioResource Project.

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