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Systematic Characterization of Human 21st Chromosome Orthologs in *Caenorhabditis elegans*

SARAH K. NORDQUIST*, ALLISON GRIFFITH[§], JONATHAN T. PIERCE *[,§]

*Institute for Neuroscience, [§] Institute for Cellular and Molecular Biology, [§] Center for Learning and Memory, [§] Waggoner Center for Alcohol & Addiction Research, Department of Neuroscience, The University of Texas at Austin, TX, 78712

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[§]To whom correspondence should be addressed:

Jonathan T. Pierce

University of Texas at Austin

Department of Neuroscience

2506 Speedway NMS 5.234

Mailcode C7350

Austin, TX 78712

E-mail: jonps@austin.utexas.edu

Phone: 512-232-4137

ABSTRACT

Individuals with Down syndrome have neurological and muscle impairments due to an additional copy of the human 21st chromosome (HSA21). HSA21 is conservatively estimated to contain 213 genes that encode protein. Only a few of these genes have been linked to specific Down syndrome phenotypes, while the remainder are understudied. To gain insight into the *in vivo* roles of these genes, we studied loss-of-function phenotypes of all HSA21 orthologs in the nematode *Caenorhabditis elegans*. Excluding the expansion of keratin genes, 52% of HSA21 genes have a potential ortholog in *C. elegans*. Knock-down using RNA interference of 26% of HSA21 orthologs yielded phenotypes suggestive of neuronal and/or muscle dysfunction. Additionally, four genes were identified in an RNAi screen for decreased acetylcholine transmission. We conducted further study of synaptic transmission and neuromuscular function by quantitative analysis of several behaviors for defined HSA21 orthologs with loss-of-function mutants. Mutations in four genes caused defects in acetylcholine secretion. These include orthologs of *NCAM2* (*ncam-1*), *SYNJ1* (*unc-26*), *PDXK (pdxk-1)* as well as for a poorly characterized gene, *N6AMT1* (*mtq-2*). As the first systematic functional analysis of HSA21 genes, this study may serve as a platform to understand genes that underlie phenotypes associated with Down syndrome.

ARTICLE SUMMARY

Down syndrome causes neurological and muscle dysfunction due to an extra 21st chromosome. This chromosome has over 200 genes, most of which are understudied. To address this, we studied whether reducing function of these gene equivalents in the worm *C. elegans* caused neuronal or muscle defects. We found evidence that about one quarter of genes conserved between human and worm may function in neurons, muscle, or both. Among these, the highly conserved but uncharacterized neuronal gene, *mtq-2*, was found to be important for synaptic transmission. Our analysis may reveal novel functions of genes that cause problems in 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). In addition to varying degrees of intellectual disability, including learning and memory problems, individuals with DS experience additional 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 walking efficiently (Pitetti, Climstein, Mays, & Barrett, 1992). Furthermore, nearly all people with DS develop Alzheimer's-like dementia upon reaching middle age (Coyle, Oster-Granite, & Reeves, 1988).

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). However, research on rare individuals with partial trisomy 21 has shown that the amount of extraneous genetic material does not easily 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, Richtsmeier, Leszl, & Reeves, 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 complement of DS-associated phenotypes (N. P. Belichenko et al., 2009; Olson et al., 2004). Aside from the DSCR mouse model, additional mouse models of DS trisomic for different syntenic regions of HSA21 have been instrumental in the analysis of DS-related phenotypes including craniofacial defects (Richtsmeier, Baxter, & Reeves, 2000; Richtsmeier, Zumwalt, Carlson, Epstein, & Reeves, 2002), cerebellar cell loss (Baxter, Moran, Richtsmeier, Troncoso, & Reeves, 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 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 also offer 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 important insight into gene function. Several

HSA21 orthologous genes were initially characterized in this way in invertebrate models. For instance, early work conducted in *Drosophila* found 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 (Patil et al., 1995; Shindoh et al., 1996; Song, Chung, & Kurnit, 1997; Song et al., 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 *Drosophila*. Mutations in the *Drosophila* 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 for human disease, exhibiting 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 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 similar to those of mammals. Genetic screens using the acetylcholinesterase inhibitor aldicarb in worm have identified genes critical for synaptic function in mammal (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 may have been overlooked. The goal for our study was to determine the general *in vivo* functions of all HSA21 genes represented by orthologs in *C. elegans*. We analyzed phenotypes induced by RNA interference and loss-of-function mutations. We placed special emphasis on examining phenotypes that correlate with neuronal and/or muscle function. Disruption of several uncharacterized HSA21 gene orthologs in worm yielded significant neuromuscular phenotypes. This knowledge may inform predictions for which genes contribute the neuronal and muscle phenotypes associated with DS.

MATERIAL AND METHODS

Animals

C. elegans were grown on NGM (nematode growth media) agar plates seeded with OP50 bacteria at 20 °C as described (Brenner, 1974b). The following strains were used: N2 Bristol as wild type, which was also used for outcrossing (Brenner, 1974); BB3 *adr-2(gv42)* III; CB211 *lev-1(e211)* IV; DR97 *unc-26(er345)* IV; EK228 *mbk-1(pk1389)* X; FX0776 *sod-1(tm776)* II; JPS636 *C24H12.5(tm3322)* II, 3X outcrossed from FX03322; JPS637 *mtq-2(tm3565)* II, 3X outcrossed from FX03565; JPS638 *cle-1(gk421)* I, 1X outcrossed from VC943; JPS639 *cysl-1(ok762)* X, 2X outcrossed from RB899; JPS641 *pad-2(tm1756)* III, 3X outcrossed from FX01756; JPS642 *set-29(ok2772)* I, 3X outcrossed from RB2097; JPS683 *itsn-1(ok268)* IV, 2X outcrossed from VC201; JPS684 *B0024.15(tm6706)* V, 1X outcrossed from FX06706; JPS685 *dip-2(ok885)* I, 1X outcrossed from RB979; JPS686 *rcan-1(tm2021)* III, 2X outcrossed from FX02021; JPS687 *igcm-1(ok711)* X, 2X outcrossed from RB870; JPS688 *Y105E8A.1(tm2626)* I, 1X outcrossed from FX02626; JPS689 *ikb-1(nr2027)* I, 1X outcrossed from NS3026; JPS690 *rnt-1(ok351)* I, 1X outcrossed from VC200; JPS691 *nrd-1(tm2657)* I, 1X outcrossed from FX02657; JPS692 *zig-10(tm6327)* II, 2X outcrossed from FX06327; JPS693 *D1037.1(ok1746)* I, 1X outcrossed from RB1489; JPS694 *mtq-2(ok3740)* II, 2X outcrossed from VC3037; VC868 *eva-1(ok1133)* I; VC40230 *ncam-1(gk525470)*; and VC40866 *pdxk-1(gk855208)*. For transgenic strains, JPS611 was generated by transforming N2 with *mtq-2p::mCherry::unc-54 UTR* at

25ng/µl to yield *vxEx611*. JPS612 was generated by transforming LX929 *vsIs48[unc17::GFP]* with *mtq-2p::mCherry::unc-54 UTR* at 25ng/µl to yield *vxEx612*. The primer sequences used for JPS611 and JPS612 are 5'-CTGAAAAAATGGAAATGTTTTTT-3' (upstream), 5' TTCTGGTCGATATGGGGTTC-3' (downstream). Both JPS610 and JPS621 strains were generated by transforming JPS637 *mtq-2(tm3565)* with *mtq-2p::mtq-2::mtq-2 UTR* at 25 ng/µl to yield *vxEx610* and *vxEx621* respectively. The primer sequences used for JPS610 and JPS621 are 5'-CAAATATTCGGGAGTAGTCTGAAAG-3' (upstream), 5'-TGATTCTAGCCAGCTACTGACTTTT-3' (downstream).

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 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 ºC, and observed for the following week. This period covers two generations

for untreated and RNAi-treated worms. 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. Control-treated worms were grown on L440 background strain bacteria that harbored an empty RNAi vector.

Phenotypic analysis

For our RNAi analysis, we used a stereomicroscope (Olympus SZX16, Japan) to score the incidence of 8 phenotypes (listed in Table 2) blind to RNAi treatment or control. In addition to the 6 phenotypes (Emb, Ste, Stp, Gro, Lva, Lvl) included in seminal RNAi screens (Fraser et al., 2000), we also noted the incidence of obvious abnormal forward or backward locomotion. To account for potential age-associated differences, locomotion phenotypes were only scored on the first day of adulthood for both the F0 and F1 generation worms. All experiments were performed at 20 ºC.

Body Bend Assay

Body bends were counted as previously described (Hart, 2006). One day before the experiment, fifteen L4-stage worms were picked to a freshly seeded plate. The following day, a single adult worm was observed by eye under a stereomicroscope for three minutes on the same plate. The observed animal was then removed to prevent analyzing the same worm multiple times. At least ten worms were observed for each genotype.

Pharyngeal Pumping Assay

Worms were synchronized by bleaching mixed-stage adult worm plates, retrieving eggs and allowing them to grow to first-day adulthood. Pumping was of each worm was quantified by eye for thirty seconds under a stereomicroscope at x100 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 were analyzed per genotype.

Aldicarb Assay

Sensitivity to the acetylcholine esterase inhibitor aldicarb was quantified as described (Mahoney, Luo, & Nonet, 2006). At least 20, day-one adult staged worms were evaluated per trial. All worms were checked at the start of the trial to ensure that they were living and mobile. 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 using 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 being prodded three times on the head and tail with a platinum wire.

Levamisole Assay

Sensitivity to the acetylcholine receptor agonist levamisole was measured as described (Lewis, Wu, Berg, & Levine, 1980). At least 20, day-one adult staged worms

were placed on plates treated with 800-μM levamisole. Worms were scored for paralysis every ten minutes for one hour. Assays were performed blind and in triplicate.

Statistical Analysis

Body bends and pharyngeal pumping values were compared with a one-way ANOVA with a Bonferroni correction. Aldicarb and levamisole responses of mutants were compared with a two-way ANOVA with a Bonferroni correction using GraphPad Prism software.

RESULTS

Determining 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 included only those proteins reviewed by SwissProt. SwissProt provides manual curation and review for each protein, which ensures high quality, non-redundant entries (Consortium, 2014). This strategy yielded a total number of 213 protein-coding genes on the 21st chromosome (Supplemental Table 1).

Determining HSA21 orthologs in C. elegans

To match worm orthologs to protein-coding genes on the human 21st chromosome, we relied on the publicly available database, OrthoList. OrthoList compiles results from a meta-analysis of four orthology prediction programs—Ensembl Compara, OrthoMCL, InParanoid, and Homolgene (Shaye & Greenwald, 2011).

OrthoList predicts at least one worm ortholog for 85 of the 213 HSA21 protein-coding genes (Figure 1A). HSA21 encodes 48 predicted keratin proteins, which have no orthologous genes in worm (Shaye & Greenwald, 2011). Excluding the keratin-encoding genes from analysis, *C. elegans* has at least one orthologous gene to 52% of HSA21 genes. These 85 HSA21 genes with a potential worm ortholog are represented by 157 genes in worm. The discrepancy between the number of human genes and worm genes can be explained, in part, by the presence of several large clusters of paralogs in worm. For instance, the human gene *ABCG1* (*ATP-binding cassette, sub-family G (WHITE), member 1*) is represented by eight paralogous genes in worm; *CBS* (*cystathionine-beta-synthase*) by seven; and *CRYAA* (*crystallin, alpha A*) by four.

Functional characterization of HSA21 orthologous genes with RNAi

To gain insight into the *in vivo* function of HSA21 gene orthologs we performed a phenotypic screen following RNAi treatment. We examined all orthologous worm genes identified by OrthoList that had an available clone, which totaled 140 RNAi clones. We screened for phenotypes that have been characterized in previous screens including abnormalities in development (Fraser et al., 2000; Kamath et al., 2003), as well as additional phenotypes—abnormalities in forward or backward locomotion—that may indicate roles in the nervous system, muscle, or both. Overall, we found that RNAi treatment of 45% of the 139 genes resulted in a visible phenotype (Table 2) suggesting roles in development and/or physiology.

We also noted sterility and/or severe growth defects associated with knockdown of several genes. For instance, knockdown of *F20D12.2* (*MCM3AP*) resulted in 100%

sterility in the parent generation and knockdown of *cct-8* (*CCT8*) caused highly penetrant sterility, larval arrest, and growth defects. These phenotypes for *cct-8* and *F20D12.2* are consistent with previously published worm research (Green et al., 2011; Rual et al., 2004; Simmer et al., 2003). The mammalian CCT8 protein is a molecular chaperone that assists with protein folding in cytosol (Kubota, Hynes, & Willison, 1995). MCM3AP protein acetylates MCM3 and inhibits cell-cycle progression (Schuurs-Hoeijmakers et al., 2013). Interestingly, both *MCM3AP* and *CCT8* have been suggested as candidate genes for the development of intellectual disability, but have been understudied in mammal (Sturgeon, Le, Ahmed, & Gardiner, 2012).

In addition to morphological phenotypes, we found that RNAi treatment of 26% of HSA21 orthologs resulted in abnormalities in either forward or backward locomotion. Several of the worm genes that we identified have established roles in the nervous system, including *apl-1 (APP)* and *hlh-16* (*OLIG1 and OLIG2)* (Bertrand, Bisso, Poole, & Hobert, 2011; Ewald et al., 2012; Hornsten et al., 2007) . We also identified several genes associated with defects in locomotion that are less clearly linked to the nervous system in worm. Several of these genes, including *pad-2 (POFUT2)* and *wdr-4 (WDR4)*, are expressed in the nervous system or have evidence supporting a role in the nervous system in other model animals (see Discussion).

Thus, the results from our systematic RNAi screen of HSA21 orthologs are consistent with published phenotypes for certain genes, and suggest that many uncharacterized genes may have *in vivo* roles in nervous system development and/or function.

HSA21 orthologs required for proper synaptic function

To assess the potential role of genes in neurotransmission, we measured how RNAi treatment of HSA21 gene orthologs altered sensitivity of worms to paralysis by the acetylcholinesterase inhibitor aldicarb. Many human 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 wild-type worms by chronically activating body-wall muscle after prolonged presence of acetylcholine at the neuromuscular junction. Mutants defective in acetylcholine release, for instance, show resistance to paralysis by aldicarb, due to decreased levels of acetylcholine in the cleft (Mahoney et al., 2006). Conversely, mutations that increase acetylcholine secretion often lead to increased sensitivity to paralysis by aldicarb (Gracheva et al., 2006; McEwen, Madison, Dybbs, & Kaplan, 2006). We identified four genes that caused significant resistance to paralysis by aldicarb when knocked down with RNAi (Figure 2A, $p < 0.001$, one-way ANOVA, Bonferroni post-hoc correction). These genes included: *rcan-1* (*RCAN1*), *ncam-1* (*NCAM2*), *mtq-2* (*N6AMT1*), and *pdxk-1* (*PDXK*).

As a complementary strategy to RNAi, we also tested aldicarb sensitivity for HSA21 orthologs with viable mutants carrying predicted loss-of-function alleles. To focus our analysis, we examined only mutants representing orthologs identified by the InParanoid algorithm with any relationship except many-to-many (Figure 1B) (Sonnhammer & Östlund, 2015). We excluded from analysis many-to-many orthologs, which may be less useful in predicting function in human (Altenhoff, Studer, Robinson-Rechavi, & Dessimoz, 2012).

None of the mutants showed significant hypersensitivity to 1-mM aldicarb over a 3-hour time course (data not shown). However, we noted two mutants, *unc-26* and *mtq-*

2, that displayed significant resistance to aldicarb (Figure 2B). To independently test whether loss of function *ncam-1* and *pdxk-1* genes caused resistance to aldicarb, we sought characterized loss-of-function mutants but found none available. Therefore, we tested alleles with putative loss-of-function mutations in conserved residues. We found that *ncam-1(gk525470)* and *pdxk-1(gk855208)* mutants displayed resistance to aldicarb, consistent with our RNAi results.

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 to levamisole, a potent agonist for the nicotinic cholinergic receptor (Lewis et al., 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 defective in cholinergic receptor signaling or muscle function. We found that *mtq-2*, *ncam-1*, and *pdxk-1* mutants displayed wild-type-like or hypersensitive responses to levamisole (Figure 4). These results suggest that their resistance to aldicarb is due to defective presynaptic release of neurotransmitter.

Although *unc*-26, the worm ortholog of *SYNJ1*, has an established role in synaptic signaling (Harris, Hartwieg, Horvitz, & Jorgensen, 2000), a synaptic role for *mtq-2* has never been described. To better link the *mtq-2* mutation with the aldicarb resistance phenotype, we found that an independent predicted null mutant *mtq-2(ok3740)* also showed significant resistance to aldicarb (Figure 3A) and wild-type-like sensitivity to levamisole (Figure 4A). We rescued aldicarb sensitivity of the *mtq-2*(*tm3565*) mutant with two independent strains expressing *mtq-2* genomic DNA (Figure

5A). To determine if *mtq-2* may influence synaptic function in neurons, we generated a transcriptional reporter and found that it expressed mCherry in the adult nervous system (Figure 5B). Specifically, mCherry was observed throughout the nerve ring and ventral cord neurons and co-expressed with cholinergic neurons expressing GFP (Figure 5B,C). Together, these results support the idea that *mtq-2* may be required in neurons for normal release of acetylcholine throughout the nervous system.

HSA21 orthologs important for other neuromuscular behaviors

To search for additional HSA21 gene orthologs that might have roles in the nervous system not revealed by our aldicarb screen and phenotypic RNAi screen, we also assayed the body bending of 22 mutants during crawling. Mutations in single genes related to the neuromuscular system often cause defective locomotion. As expected from previous studies (Brenner, 1974a), we found that loss of *unc-26 (SYNJ1)* led to severe lack of coordination and significantly decreased body bending. Mutation in all other genes had no significant effect on body bending; however, we noted some subtle, qualitative differences among mutants (Supplemental Table 2).

As another measure of neuromuscular function, we next assessed feeding behavior. *C. elegans* pumps bacterial food into its gut through a muscular feeding organ called a pharynx. Defects in pharyngeal pumping have helped to identify vesicular glutamate transporters (e.g. *eat-4*) and nicotinic cholinergic receptor subunits (Avery, 1993). We observed significant reductions in pharyngeal pumping in eight loss-of-function mutants: *unc-26 (SYNJ1), B0024.15 (B3GALT5), mtq-2 (N6AMT1), pad-2 (POFUT2), cle-1 (COL18A1), cysl-1 (CBS), sod-1 (SOD1),* and *set-29 (SETD4)* (Figure

6A). Of the genes that we identified with pharyngeal pumping deficits (not previously linked to pharyngeal pumping), several had overlapping measures of neuromuscular dysfunction in aldicarb sensitivity and/or locomotion. Most notably, *mtq-2* mutants showed reduced pharyngeal pumping that was rescuable, further suggestive of a role in the nervous system (Figure 6B).

DISCUSSION

Our research advances functional annotation of genes on the human 21st chromosome by characterizing neuronal roles of orthologs in *C. elegans*. Though there have been several rigorous, large-scale reverse genetics screens in *C. elegans*, they have focused less explicitly on neuronal phenotypes (Fraser et al., 2000; Kamath et al., 2003), or have focused on screening other genes selected for their predicted roles in the nervous system (Sieburth et al., 2005; Vashlishan et al., 2008). Within the field of Down syndrome, previous research has focused on the functions of contiguous groups of genes, such as those included in the DSCR (X. Jiang et al., 2015), but the vast majority of HSA21 genes remain functionally uncharacterized. Our results suggest that orthologs of some of these uncharacterized genes have a neuronal role in worm and, therefore, may contribute to the cognitive and motor phenotypes seen in Down syndrome.

Using RNAi, we found that one quarter of HSA21 gene orthologs may have roles in neurons and/or muscle (Tables 1&2). We identified several genes with qualitative roles in locomotion, some previously implicated in nervous system function, including *apl-1* (APP) and *hlh-16* (OLIG1 and OLIG2). In worm, *apl-1* (amyloid precursor like) is

an essential gene that functions within the nervous system (Hornsten et al., 2007). Its closest human HSA21 ortholog, *APP* (amyloid precursor protein) is a key gene associated with the development of the neurodegenerative disorder Alzheimer's disease, including in people with Down syndrome and in a DS mouse model (Salehi et al., 2006). The worm gene, *hlh-16* (helix loop helix), encodes a basic helix-loop-helix (bHLH) transcription factor as do its predicted human orthologs, *OLIG1*/2. In worm, *hlh-16* is an asymmetrically expressed gene that mediates axonal extension of a specific neuron, AIY, and may be involved in its fate specification (Bertrand et al., 2011). Similarly, the mammalian genes *OLIG1/2* specify oligodendrocyte and motor neuron fate in the central nervous system (Lu et al., 2002; Zhou & Anderson, 2002; Zhou, Wang, & Anderson, 2000).

Additionally, we observed locomotion defects following RNAi treatment of genes with less clearly defined functional roles in the nervous system, including *pad-2 (POFUT2)* and *wdr-4 (WDR4)*. In worm, loss of *pad-2* results in defects in ventral nerve cord and neuron morphology (Menzel et al., 2004). In mouse, homozygous loss of the *pad-2* ortholog *Pofut2* results in embryonic lethality, which precludes study in the nervous system (Du et al., 2010). Based on sequence homology, *wdr-4* (WDR4) is predicted to encode a WD repeat protein. In mouse, *WDR4* expresses most strongly in fetal tissue, including brain, kidney, and heart (Michaud et al., 2000). Further, expression of the mammalian ortholog of *wdr-4*, *WDR4*, is tightly regulated and has been proposed as a potential candidate gene contributing to DS phenotypes (Sultan et al., 2007).

Genes that we identified as having roles in locomotion are alluring candidates for nervous system development and/or function. Given the small number of neurons in the worm nervous system, reduced function of a single gene often causes readily observable abnormalities in locomotion. For instance, many genes within the Unc (unc<u></u>coordinated) class play pivotal roles in either nervous system function and/ or development: *unc-10* (RIM) and *unc-31* (CAPS) mediate synaptic vesicle exocytosis, while homeobox genes such as *unc-4* specify neuronal cell fate (Hobert, 2010; Richmond, 2005).

However, a negative locomotion phenotype following RNAi treatment in *C. elegans* must be interpreted with the following caveats in mind: 1) RNAi does not efficiently abolish all gene activity, and results from RNAi treatment are, thus, more variable than results gleaned from analysis of mutants. 2) Genes that play important roles in nervous system function do not necessarily display locomotion abnormalities when mutated. For instance, the *rab-3* mutant exhibits defects in synaptic transmission and synaptic vesicle density, yet move essentially as wild type (Nonet et al., 1997). In this screen, we did not observe locomotion abnormalities following RNAi treatment of *mtq-2*, yet found robust synaptic and neuromuscular phenotypes in loss-of-function mutants. 3) Disruption of genes that are functionally compensated by other genes often yields no observable phenotype.

We also identified orthologs that appeared to have more specific roles in synaptic transmission as determined by aldicarb screening. These include *unc-26 (SYNJ1), pdxk-1 (PDXK), ncam-1 (NCAM2),* and *mtq-2 (N6AMT1)*. Our finding that all four mutants were effectively paralyzed by the acetylcholine receptor agonist levamisole

suggests that they are deficient in release of acetylcholine rather than receiving acetylcholine signal at the neuromuscular junction. Thus, they likely have presynaptic roles in the nervous system.

Consistent with this idea, the worm ortholog of synaptojanin, *unc-26* (*SYNJ1*), is involved in synaptic vesicle recycling and *unc-26* loss-of-function mutant worms have been previously been found to be resistant to aldicarb (Harris et al., 2000). *NCAM2* (neural cell adhesion molecule) has been previously linked to synaptic function in mammals. Activation of *NCAM2* has been shown to regulate neurite branching in mouse cell neuron culture, which hints at a role for *NCAM2* when overexpressed in the context of Down syndrome (Sheng, Leshchyns'ka, & Sytnyk, 2015). In mammals, *NCAM2* has been shown to be involved in axon pathfinding in olfactory neurons and, more recently, has been shown to be enriched in synapses in the human hippocampus (Leshchyns'ka et al., 2015). Biochemically, 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 been studied well in mammal (Mouse Genome Informatics and the International Mouse Phenotyping Consortium (IMPC)).

To our knowledge, MTQ-2 has not been implicated previously in nervous system function. 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 (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 additional substrates *in vitro* and *CHD5* (chromodomain helicase DNA-binding protein 5) and *NUT* (nuclear protein in testis) *in vivo* (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 *mtq-2* widely expresses throughout the nervous system and, specifically, in cholinergic neurons, but not other tissues. 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 may share a conserved function in synaptic transmission. *C. elegans* may provide an especially informative system to study *mtq-2* since deletion of *N6AMT1* causes post-embryonic lethality in mice (Liu et al., 2010).

We found evidence for roles in other neuromuscular behaviors for certain HSA21 gene orthologs. Several among these genes have established roles in neuromuscular signaling. For instance, *cle-1 (COL18A1)* encodes type XVIII collagen involved in synaptogenesis and cholinergic transmission (Ackley et al., 2001; 2003). The gene *cysl-1 (CBS)* functions in neurons and is expressed throughout the nervous system, including the pharyngeal nervous system (Ma, Vozdek, Bhatla, & Horvitz, 2012). The neuromuscular phenotypes resulting from loss of these genes above argue for their

prioritization for future studies seeking to gain insight into neuromuscular dysfunction in DS.

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; 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 et al., 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 with further investigation using traditional mouse models and human cell line approaches.

Supplemental information: Supplemental information found online

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Figure 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, over half of the remaining 165 genes have orthologs in *C. elegans*. All 85 putative orthologs in worm (blue and green) with an available RNAi clone were tested in the RNAi phenotypic screen (Table 1-2). (**B**) Representation of InParanoid-defined orthologs (human to worm). All orthologs in any relationship except many-to-many were tested in all subsequent behavioral assays (Fig-1-6). For 1-to-many orthologs, the *C. elegans* gene with the highest InParanoid bootstrap score was tested.

Table 1: Gross phenotypes arising from RNAi treatment of HSA21 orthologs. *C. elegans* orthologs are defined by OrthoList (157 worm genes representing 85 human genes). Blue text indicates an InParanoid-defined ortholog. Bold text indicates an InParanoid-defined 1-to-1 ortholog (19 worm genes representing 19 human genes). A dash indicates no bacterial RNAi clone in the Ahringer library, and nd indicates no growth of RNAi bacterial clone. Abbreviations for phenotypes are as follows: Emb (embryonic lethal), Ste (sterile), Stp (sterile progeny), Gro (post-embryonic slow growth), Lva (larval arrest), Lvl (larval lethal), PE (post-embryonic), Lv (locomotion variant), and Blv (backward locomotion variant).

Table 2: Summary of gross phenotypes arising from RNAi treatment of HSA21 orthologs with RNAi. All percentages reflect the incidence of given phenotype out of a total of 139 RNAi clones tested. Clones were tested in triplicate in a two-generation screen.

Figure 2: Screen for aldicarb resistance. Black shading of bars indicates a control. Gray shading of bars corresponds to level of significance relative to control: light gray (not significant); dark gray ($p < 0.001$). (**A**) Aldicarb resistance of defined orthologs in presence of RNAi treatment. RNAi treatment of *mtq-2, rcan-1, ncam-1,* or *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 Bonferroni 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). (**B**) Aldicarb resistance of mutants. Ratio of moving worms at 180 minutes in 1-mM aldicarb (n = 3 trials, 20-25 worms per trial). The following alleles displayed significant ($p < 0.001$, one-way ANOVA with Bonferroni post-hoc correction) resistance relative to WT: *pdxk -1(gk855208), mtq-2(tm3565), mtq-2(ok3740), ncam-1(gk525470),* and *unc-26(e345)*. Wild-type control was run in parallel (n = 33 trials). HIC (hypersensitive to inhibitors of cholinesterase) control was *dgk-1(sy48)* run in parallel (n = 33 trials*)*. Error bars indicate SEM.

Figure 3. Response of deletion and putative loss-of-function mutants to aldicarb. Time course to paralysis on 1-mM aldicarb for resistant mutants from Figure 2B (n = 5-6 trials, 20-25 worms per trial). Wild-type control was run in parallel (n = 33 trials). HIC (hypersensitive to inhibitors of cholinesterase) control was *dgk-1(sy48)* run in parallel (n = 33 trials*)*. All mutants in panels A-D (shown with white symbols) had significantly reduced responsiveness to aldicarb

relative to WT control ($p < 0.001$, two-way ANOVA with Bonferroni post-hoc correction). Error bars indicate SEM.

Figure 4. Response of known and putative loss-of-function mutants to levamisole. Time course of paralysis in the presence of 800-μM levamisole (n=3 trials, 25-30 worms per trial). Positive control was *lev-1(e211)*. (**A**) Neither *mtq-2(tm3565)* nor *mtq-2(ok3740)* displayed significant differences in levamisole response relative to WT. (**B**) *unc-26(e345)* was significantly ($p < 0.001$) hypersensitive to levamisole. (**C**) *ncam-1(gk525470)* displayed a WT-like levamisole response. (**D**) *pdxk-1(gk855208)* was significantly hypersensitive ($p < 0.001$) to levamisole. Significance was determined with two-way ANOVA with Bonferroni post-hoc correction. Error bars indicate SEM.

Figure 5. *mtq-2* functions in the nervous system. (**A**) Expression of extrachromosomal array spanning 5.5 kb of genomic DNA independently rescued aldicarb resistance of *mtq-2 (tm3565)* for two strains. For the *mtq-2* associated strains, n = 6 trials, 20-25 worms per trial. Wild-type control was run in parallel (n = 33 trials). Error bars are SEM. (**B**) A transcriptional reporter of *mtq-2* expressed mCherry in the adult nervous system. Scale bar, 100μm. (**C**) A transcriptional reporter of *mtq-2* expressed mCherry in cholinergic neurons labeled with GFP. Scale bar, 50μm.

Figure 6: Screen for pharyngeal pumping defects. (A) Pumps per minute in the presence of standard bacterial food. Mutants shaded in dark gray displayed significant reductions in pharyngeal pumping ($p < 0.001$). For all mutants, n = 30. Wild-type (WT) control is Bristol N2 run in parallel (n = 290). Negative control is *dgk-1(sy48* run in parallel (n = 290). Error bars indicate SEM. **(B)** An extrachromosomal array spanning the *mtq-2* locus restores pharyngeal pumping deficits of *mtq-2(tm3565)* to near WT levels. Wild-type (WT) control is Bristol N2 run in

parallel (n = 290). For *mtq-2(tm3565)* and transgenic rescue lines (JPS610 (*mtq-2(tm3565); mtq-2(+) #1)* and JPS621 (*mtq-2(tm3565); mtq-2(+) #2)*), n = 30. Error bars indicate SEM.

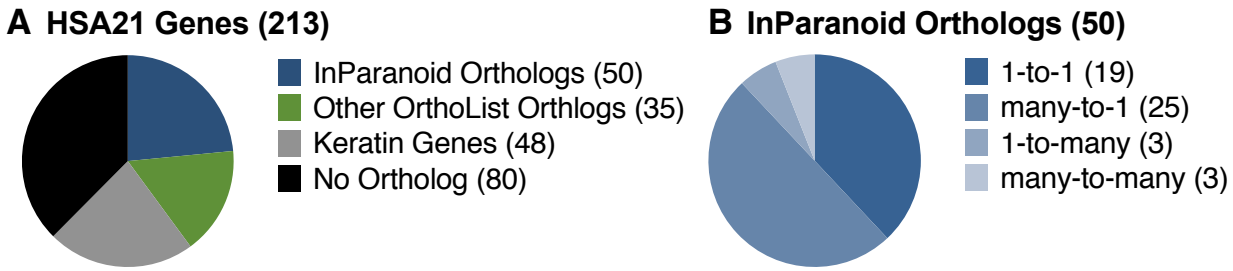

A HSA21 Genes (213)
InParanoid Orthologs (50)
Other OrthoList Orthlogs (35)
Keratin Genes (48)
No Ortholog (80)
B InParanoid Orthologs (50)
1-to-1 (19)
many-to-1 (25)
1-to-many (3)
many-to-many (3)

Table 1. Gross phenotypes arising from RNAi treatment of HSA21 orthologs									
Human Gene	**Worm Sequence ID**	**Worm Gene**	**RNAi Clone**	**Phenotype Detected**	**Emb**	**Ste, Stp**	**Gro, Lva**	**Lvl**	**Blv, Lv**
ABCG1	C05D10.3	*wht-1*	III-3O09	✓	Emb		Gro		
	F02E11.1	*wht-4*	II-3G07						
	F19B6.4	*wht-5*	IV-6F21	✓			Gro		
	Y42G9A.6	*wht-7*	III-3O17	✓					Blv
	C10C6.5	*wht-2*	IV-6I23	✓					Blv, Lv
	C16C10.12	*wht-3*	III-2G05						
	T26A5.1	*wht-6*	III-3M20						
	Y47D3A.11	*wht-8*	III-5N24	✓					Blv, Lv
ADAMTS1	F25H8.3	*gon-1*	IV-5M07	✓		Ste			Blv
ADAMTS5	F25H8.3	*gon-1*	IV-5M07	✓		Ste			Blv
ADARB1	T20H4.4	*adr-2*	III-3J14						
APP	C42D8.8	*apl-1*	X-2N22	✓		Ste	Lva		Blv
ATP5O	**F27C1.7**	***atp-3***	**I-2O16**	✓		Ste	Lva		
B3GALT5	B0024.15	*B0024.15*	V-7K15						
	Y39E4B.9	*bre-2*	-						
	C47F8.3	*C47F8.3*	I-6M19						
	C47F8.5	*C47F8.5*	I-6M23	✓					Lv
	C47F8.6	*C47F8.6*	I-6O01						
	C54C8.3	*C54C8.3*	I-6E10						
	E03H4.11	*E03H4.11*	I-6C24						
	F14B6.4	*F14B6.4*	I-6I11						
	F14B6.6	*F14B6.6*	I-6I15						
	T09E11.10	*T09E11.10*	I-6A02						
	T09F5.1	*T09F5.1*	V-9L22						
	T15D6.5	*T15D6.5*	I-6A14						
BACE2	Y39B6A.20	*asp-1*	V-12I20						
	T18H9.2	*asp-2*	V-6B15						
	F21F8.3	*asp-5*	V-5N12	✓					Lv
	F21F8.7	*asp-6*	V-5N20						
	C11D2.2	*C11D2.2*	IV-3I19	✓			Gro		
	C15C8.3	*C15C8.3*	V-8B15	✓					Lv
	F21F8.2	*F21F8.2*	V-5N10						
	F21F8.4	*F21F8.4*	V-5N14	nd					
	F28A12.4	*F28A12.4*	V-6M19						
	F59D6.2	*F59D6.2*	V-2B02						
	F59D6.3	*F59D6.3*	V-2B04						
	K10C2.3	*K10C2.3*	X-3O08	✓					Lv
	Y39B6A.22	*Y39B6A.22*	V-12K02	✓					Blv, Lv
	Y39B6A.23	*Y39B6A.23*	V-16J24						
	Y39B6A.24	*Y39B6A.24*	V-12K04						
	ZK384.3	*ZK384.3*	V-12O15	✓			Gro		Blv, Lv
	ZK384.6	*ZK384.6*	-						
C21orf2	F09G8.5	*F09G8.5*	III-4K14						
C2CD2	R11G1.6	*R11G1.6*	X-2I04						
CBR1	T04B2.6	*dhs-31*	IV-5A22						
CBR3	T04B2.6	*dhs-31*	IV-5A22						
CBS	C17G1.7	*cysl-1*	X-4L22						

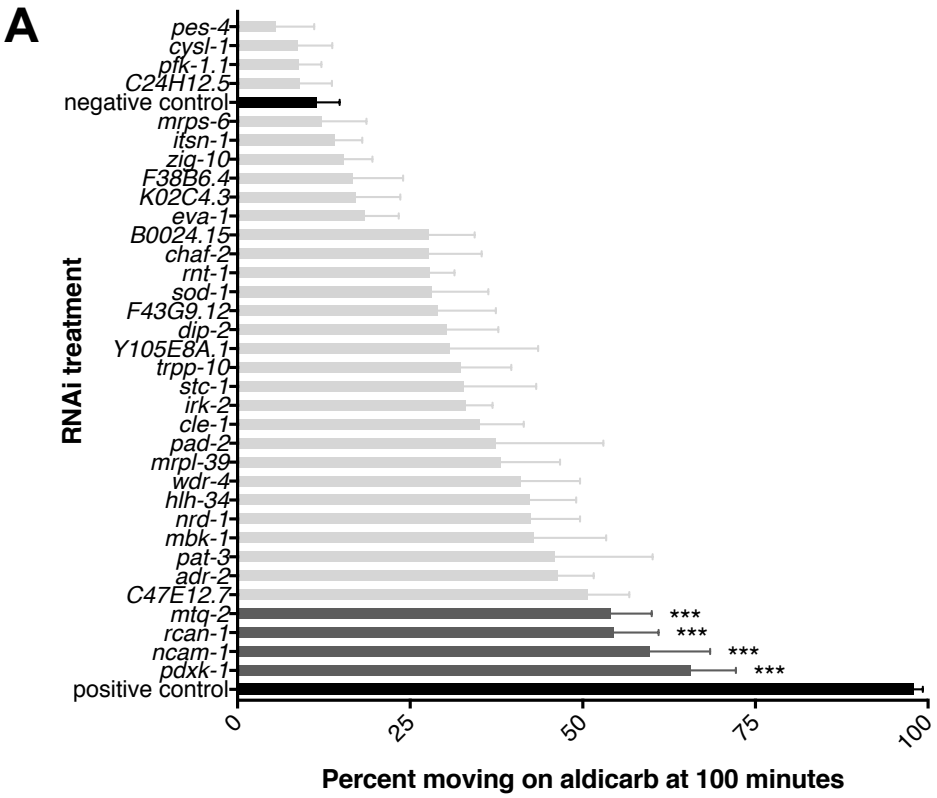
	K10H10.2	*cysl-2*	II-9M07	✓					Blv
	R08E5.2	*cysl-3*	V-3C18						
	F59A7.9	*cysl-4*	V-2A09	✓					Blv
	ZC373.1	*cbs-1*	X-8C04						
	F54A3.4	*cbs-2*	II-9B15						
	F59A7.7	*F59A7.7*	V-2A05	✓					Blv, Lv
CCT8	**Y55F3AR.3**	***cct-8***	**IV-8P07**	✓		Ste	Lva		
CHAF1B	**Y71G12B.1**	***chaf-2***	**I-8O03**						
CLIC6	D1086.9	*D1086.9*	-						
	H39E23.3	*H39E23.3*	-						
	Y105E8A.22	*exc-4*	-						
	F26H11.5	*exl-1*	II-9K13	✓					Blv
COL18A1	C36B1.1	*cle-1*	I-4E22						
COL6A1	C16E9.1	*C16E9.1*	X-3N11	✓					Lv
	C18H7.1	*C18H7.1*	IV-1A10						
	Y37B11A.1	*cutl-23*	III-8F13						
COL6A2	C16E9.1	*C16E9.1*	X-3N11	✓					Lv
	C18H7.1	*C18H7.1*	IV-1A10						
	Y37B11A.1	*cutl-23*	III-8F13						
CRYAA	T22A3.2	*hsp-12.1*	I-5G12						
	C14B9.1	*hsp-12.2*	III-4E02	✓	Emb				Lv
	F38E11.1	*hsp-12.3*	IV-4J20						
	F38E11.2	*hsp-12.6*	IV-4J22	✓					Blv
DIP2A	F28B3.4	*dip-2*	I-2M19	✓		Ste			Lv
DONSON	**C24H12.5**	***C24H12.5***	**II-1O15**						
DOPEY2	Y18D10A.13	*pad-1*	I-6F05	✓					Blv
DSCAM	F39H12.4	*igcm-1*	X-8D03	nd					
DYRK1A	T04C10.1	*mbk-1*	X-6J16						
ERG	T08H4.3	*ast-1*	II-11A17	✓					Lv
	C42D8.4	*ets-5*	X-2N18						
ETS2	F19F10.5	*ets-7*	V-5B17	✓					Blv, Lv
	C52B9.2	*ets-9*	X-2H15	✓					Blv, Lv
	F19F10.1	*F19F10.1*	V-5B09						
EVA1C	**F32A7.3**	***eva-1***	**I-7I22**						
FAM3B	M70.4	*M70.4*	IV-1L23	✓					Blv
	Y73B3A.3	*Y73B3A.3*	-						
GABPA	F19F10.5	*ets-7*	V-5B17	✓					Blv, Lv
	F19F10.1	*F19F10.1*	V-5B09						
GART	**F38B6.4**	***F38B6.4***	**X-3F13**						
GRIK1	C06E1.4	*glr-1*	III-4H05						
	B0280.12	*glr-2*	III-3D18						
	K10D3.1	*glr-3*	I-3B17						
	C06A8.9	*glr-4*	II-5H04						
	ZC196.7	*glr-5*	V-6A06						
HLCS	F13H8.10	*bpl-1*	II-10A05						
HSPA13	**F54C9.2**	***stc-1***	**II-6A08**	✓		Ste			
ITGB2	ZK1058.2	*pat-3*	III-1P02	✓		Ste			Blv
ITSN1	Y116A8C.36	*itsn-1*	IV-8I23	✓					Blv
JAM2	T25D10.2	*zig-10*	II-5A05						
KCNJ6	M02A10.2	*irk-2*	X-1I21						

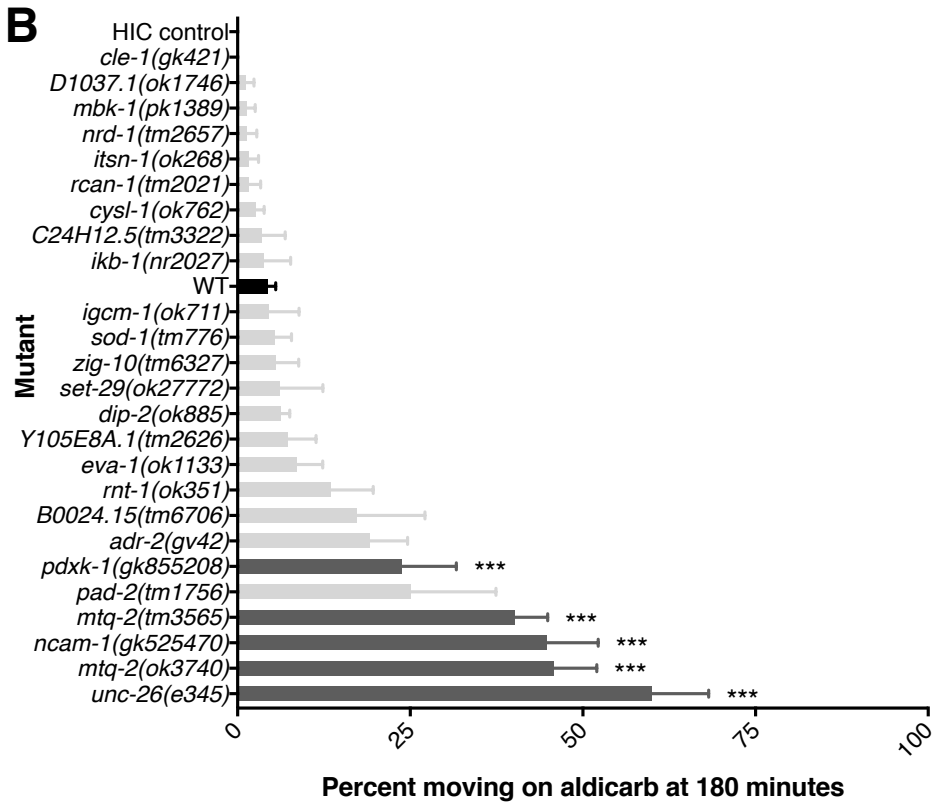
KCNJ15	K04G11.5	*irk-3*	X-6P11						
LTN1	**Y54E10A.11**	***Y54E10A.11***	**-**						
MCM3AP	F20D12.2	*F20D12.2*	IV-4E03	✓	Emb	Ste			
MORC3	ZC155.3	*morc-1*	III-2P19						
MRPL39	**Y46H3A.7**	***mrpl-39***	**V-1L20**	✓	Emb				Lv
	C47D12.6	*tars-1*	II-7P06	✓		Ste			
MRPS6	**R12E2.12**	***mrps-6***	**I-1H04**	✓		Ste	Gro		
MX1	C02C6.1	*dyn-1*	X-7K09	✓		Ste	Lva		Lv
MX2	C02C6.1	*dyn-1*	X-7K09	✓		Ste	Lva		Lv
N6AMT1	**C33C12.9**	***mtq-2***	**II-2M20**						
NCAM2	F02G3.1	*ncam-1*	X-1I17	✓					Blv, Lv
OLIG1	DY3.3	*hlh-16*	I-4I02	✓					Lv
OLIG2	DY3.3	*hlh-16*	I-4I02	✓					Lv
PAXBP1	F43G9.12	*F43G9.12*	I-4C12						
PCBP3	Y119D3B.17	*pes-4*	III-8I07	✓				Lvl	
PCNT	ZC8.4	*lfi-1*	X-2L12	✓					Lv
PDXK	**F57C9.1**	***pdxk-1***	**I-2K05**						
PFKL	Y71H10A.1	*pfk-1.1*	X-2C11	✓	Emb				
	C50F4.2	*pfk-1.2*	V-6N01						
POFUT2	**K10G9.3**	***pad-2***	**III-8G24**	✓					Blv, Lv
POTED	C04F12.3	*ikb-1*	I-4H10	✓			Gro		
	T04C12.6	*act-1*	V-7H09	✓			Lva	Lvl	
	T04C12.5	*act-2*	V-7H07	✓			Lva	Lvl	
	T04C12.4	*act-3*	V-7H05	✓			Lva	Lvl	
	M03F4.2	*act-4*	X-2J16	✓					Blv, Lv
	T25C8.2	*act-5*	III-6D08	✓		Ste	Gro, Lva		Blv, Lv
PTTG1IP	C37C3.12	*C37C3.12*	V-15K19						
PWP2	F55F8.3	*F55F8.3*	I-2H21	✓		Ste			Blv, Lv
RBM11	Y37D8A.21	*Y37D8A.21*	III-6O18						
RCAN1	F54E7.7	*rcan-1*	III-3A09						
RRP1	C47E12.7	*C47E12.7*	IV-5O03	✓		Ste	Gro		Blv, Lv
RRP1B	C47E12.7	*C47E12.7*	IV-5O03	✓		Ste	Gro		Blv, Lv
RUNX1	B0414.2	*rnt-1*	I-2L23						
SCAF4	D1007.7	*nrd-1*	I-2A21	✓		Stp			
SETD4	**Y92H12BR.6**	***set-29***	**-**						
SH3BGR	**Y105E8A.1**	***Y105E8A.1***	**I-9O01**	✓					Blv
SIM2	T01D3.2	*hlh-34*	V-9C07	✓					Blv, Lv
SLC19A1	C06H2.4	*folt-1*	V-15E08	✓					Lv
	F37B4.7	*folt-2*	V-2J03	nd					
	C50E3.16	*folt-3*	-						
SLC37A1	T10C6.6	*T10C6.6*	V-10L17						
	T11G6.2	*T11G6.2*	IV-5B02	✓	Emb				
	T11G6.3	*T11G6.3*	IV-9C04						
	T11G6.4	*T11G6.4*	IV-9C05	✓					Blv
SOD1	C15F1.7	*sod-1*	II-5G04						
	F55H2.1	*sod-4*	III-5E21	✓					Blv
	ZK430.3	*sod-5*	II-3F14	✓					Lv
SON	**D1037.1**	***D1037.1***	**I-1F05**	nd					
	C04G2.8	*C04G2.8*	IV-5E06						

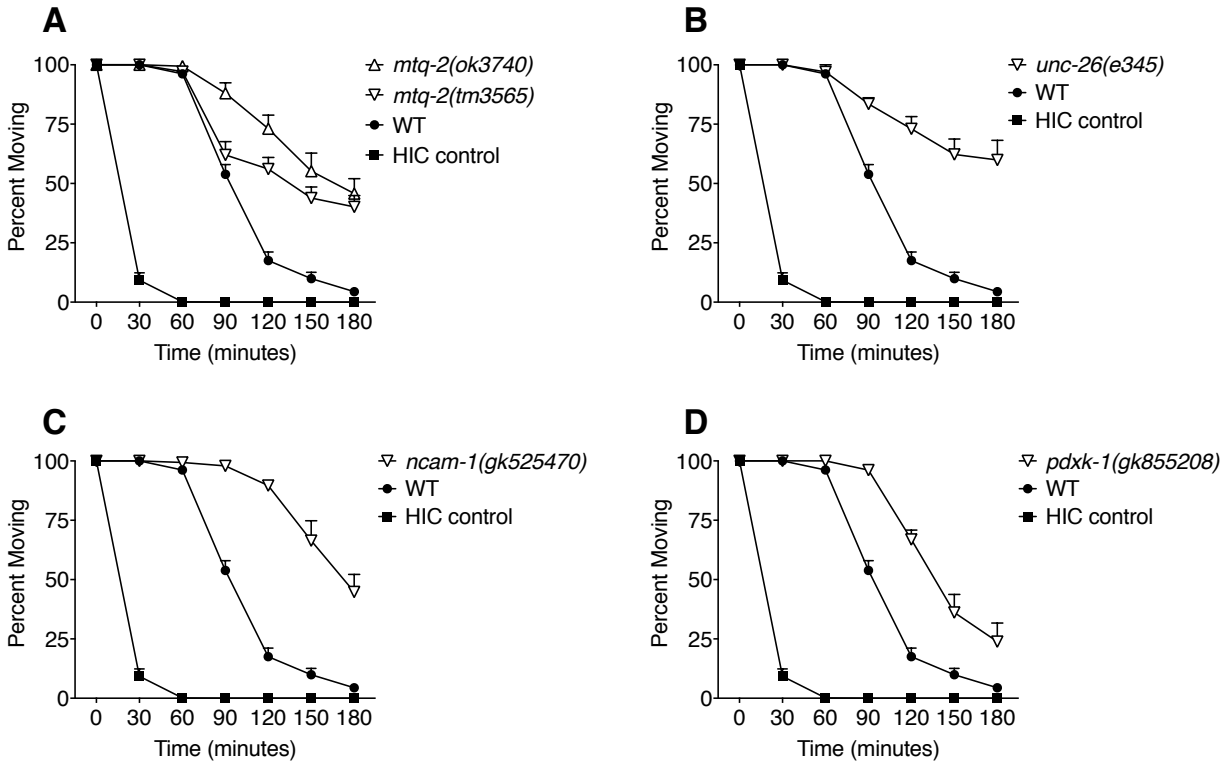
	C10G11.9	*C10G11.9*	I-3C03						
	T27A3.4	*T27A3.4*	I-2J16						
SUMO3	K12C11.2	*smo-1*	I-1O13	✓		Ste			Lv
SYNJ1	JC8.10	*unc-26*	IV-7E19						
TIAM1	C11D9.1	*cgef-2*	I-1P08						
TMEM50B	Y74C10AL.2	*Y74C10AL.2*	-						
TRAPPC10	**Y71G12A.2**	***trpp-10***	**I-9P01**						
TRPM2	ZK512.3	*ced-11*	III-8A04						
TTC3	C09E7.7	*C09E7.7*	-						
	C09E7.8	*C09E7.8*	III-3B13	✓					Blv, Lv
	C09E7.9	*C09E7.9*	-						
	F27B3.5	*F27B3.5*	-						
U2AF1	Y116A8C.35	*uaf-2*	IV-8I21	✓			Gro, Lva	Lvl	Blv, Lv
UBASH3A	F09C12.8	*F09C12.8*	-						
	F55A11.11	*F55A11.11*	-						
	T07F12.1	*T07F12.1*	X-2H18	✓	Emb				
UBE2G2	**Y87G2A.9**	***ubc-14***	**-**						
	F58A4.10	*ubc-7*	III-5K23						
URB1	T05H4.10	*T05H4.10*	-						
USP25	K02C4.3	*K02C4.3*	II-5P18						
WDR4	**Y102E9.2**	***wdr-4***	**III-3F21**	✓					Lv

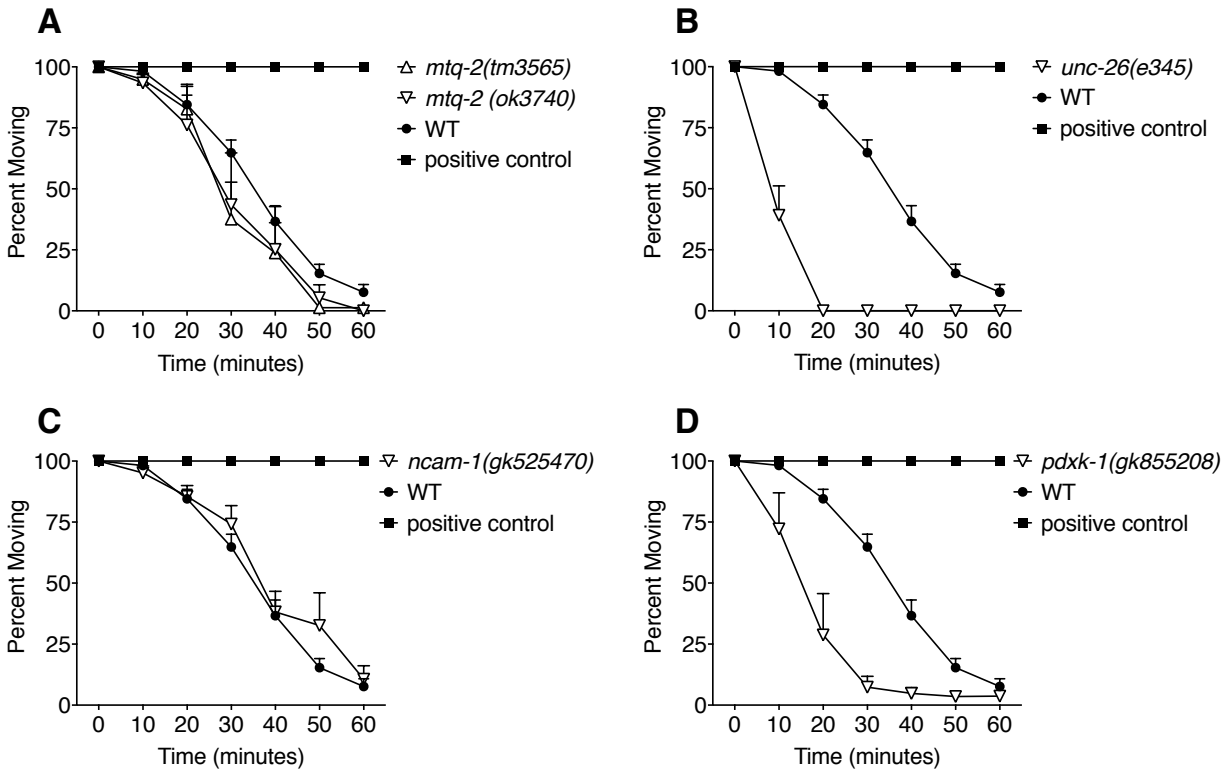
Table 2: Summary of gross phenotypes arising from RNAi treatment of HSA21 orthologs

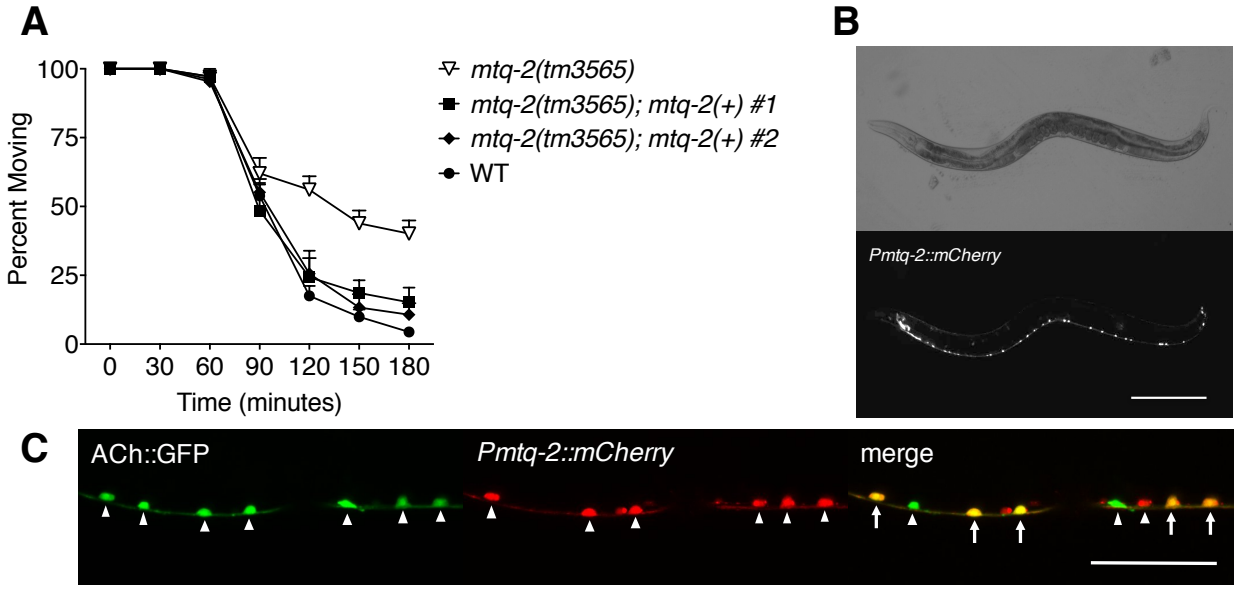
Phenotype		Number	Percent
Embryonic lethal	Emb (Embryonic lethal)	7	5.1
Sterile	Ste (Sterile)	15	11.0
	Stp (Sterile progeny)	1	0.7
Developmental delay	Gro (Growth)	9	6.6
	Lva (Larval arrest)	9	6.6
Larval lethal	Lvl (Larval lethal)	5	3.7
Post-embryonic	Lv (Locomotion variant)	32	23.5
	Blv (Backward locomotion variant)	30	22.1
Any detected		64	45.7
None detected		76	54.3


A
RNAi treatment
pes-4
cysl-1
pfk-1.1
C24H12.5
negative control
mrps-6
itsn-1
zig-10
F38B6.4
K02C4.3
eva-1
B0024.15
chaf-2
rnt-1
sod-1
F43G9.12
dip-2
Y105E8A.1
trpp-10
stc-1
irk-2
cle-1
pad-2
mrpl-39
wdr-4
hlh-34
nrd-1
mbk-1
pat-3
adr-2
C47E12.7
mtq-2
rcan-1
ncam-1
pdxk-1
positive control
***
0
25
50
75
100
Percent moving on aldicarb at 100 minutes

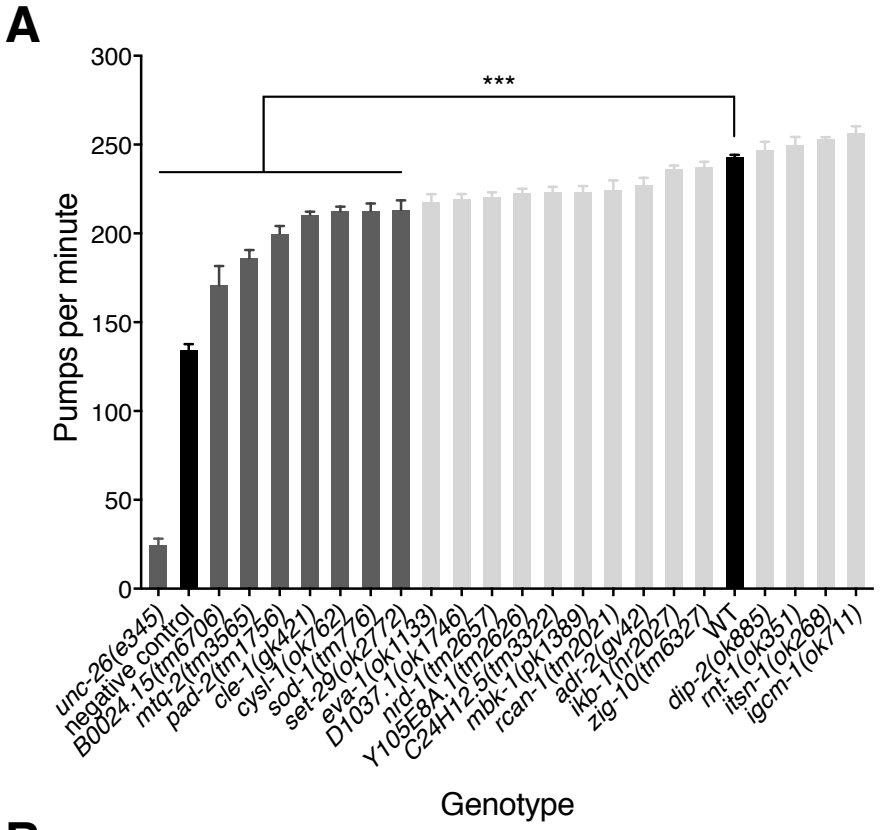

B
Mutant
HIC control
cle-1(gk421)
D1037.1(ok1746)
mbk-1(pk1389)
nrd-1(tm2657)
itsn-1(ok268)
rcan-1(tm2021)
cysl-1(ok762)
C24H12.5(tm3322)
ikb-1(nr2027)
WT
igcm-1(ok711)
sod-1(tm776)
zig-10(tm6327)
set-29(ok27772)
dip-2(ok885)
Y105E8A.1(tm2626)
eva-1(ok1133)
rnt-1(ok351)
B0024.15(tm6706)
adr-2(gv42)
pdxk-1(gk855208)
pad-2(tm1756)
mtq-2(tm3565)
ncam-1(gk525470)
mtq-2(ok3740)
unc-26(e345)
***
0
25
50
75
100
Percent moving on aldicarb at 180 minutes

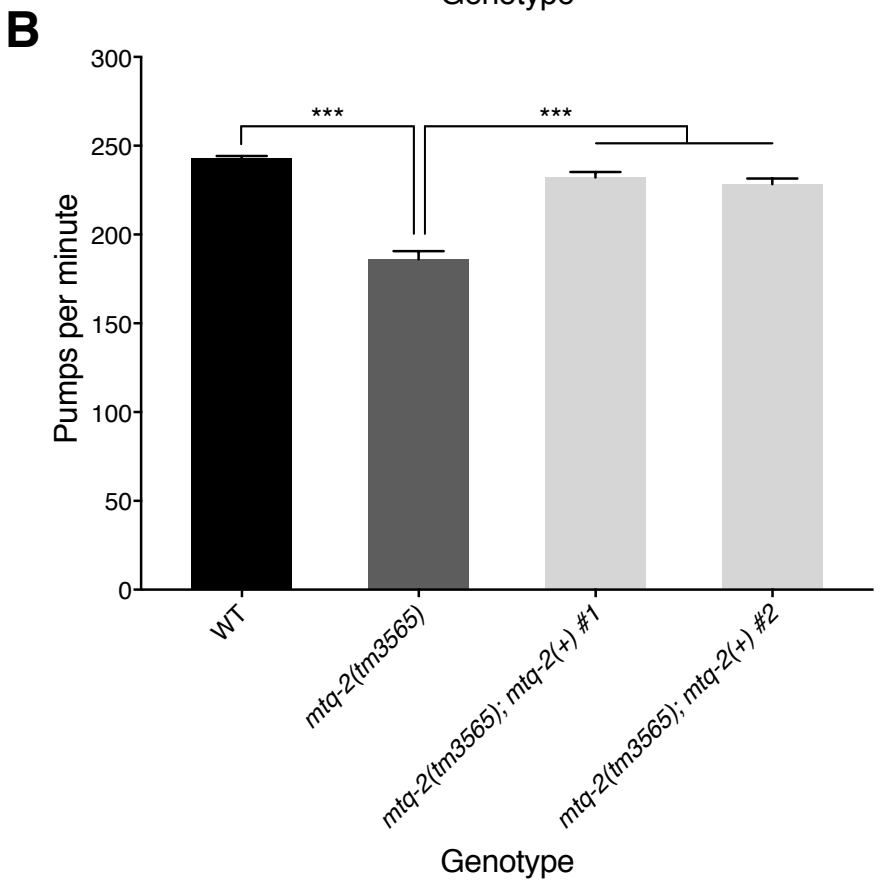

A
Percent Moving
100
75
50
25
0
0 30 60 90 120 150 180
Time (minutes)
mtq-2(ok3740)
mtq-2(tm3565)
WT
HIC control
B
Percent Moving
100
75
50
25
0
0 30 60 90 120 150 180
Time (minutes)
unc-26(e345)
WT
HIC control
C
Percent Moving
100
75
50
25
0
0 30 60 90 120 150 180
Time (minutes)
ncam-1(gk525470)
WT
HIC control
D
Percent Moving
100
75
50
25
0
0 30 60 90 120 150 180
Time (minutes)
pdxk-1(gk855208)
WT
HIC control


A
Percent Moving
100
75
50
25
0
0 10 20 30 40 50 60
Time (minutes)
mtq-2(tm3565)
mtq-2 (ok3740)
WT
positive control
B
Percent Moving
100
75
50
25
0
0 10 20 30 40 50 60
Time (minutes)
unc-26(e345)
WT
positive control
C
Percent Moving
100
75
50
25
0
0 10 20 30 40 50 60
Time (minutes)
ncam-1(gk525470)
WT
positive control
D
Percent Moving
100
75
50
25
0
0 10 20 30 40 50 60
Time (minutes)
pdxk-1(gk855208)
WT
positive control


A
100
75
50
25
0
Percent Moving
0 30 60 90 120 150 180
Time (minutes)
mtq-2(tm3565)
mtq-2(tm3565); mtq-2(+) #1
mtq-2(tm3565); mtq-2(+) #2
WT
B
Pmtq-2::mCherry

C
ACh::GFP
Pmtq-2::mCherry
merge


A
300
250
200
150
100
50
0
Pumps per minute
***
unc-26(e345)
negative control
B0024.15(tm6706)
mtq-2(tm3565)
pad-2(tm1756)
cle-1(gk421)
cysl-1(ok762)
sod-1(tm776)
set-29(ok2772)
eva-1(ok1133)
D1037.1(ok1746)
nrd-1(tm2657)
Y105E8A.1(tm2626)
C24H12.5(tm3322)
mbk-1(pk1389)
rcan-1(tm2021)
adr-2(gv42)
ikb-1(nr2027)
zig-10(tm6327)
WT
dip-2(ok885)
rnt-1(ok351)
itsn-1(ok268)
igcm-1(ok711)
Genotype


B
300
250
200
150
100
50
0
Pumps per minute
***
***
WT
mtq-2(tm3565)
mtq-2(tm3565); mtq-2(+) #1
mtq-2(tm3565); mtq-2(+) #2
Genotype