DISCOVERY AND CHARACTERIZATION OF NOVEL MANGANESE-SELECTIVE CATION DIFFUSION FACILITORS AS PUTATIVE ANTHELMINTHIC DRUG TARGETS IN *CAENORHABDITIS ELEGANS*

by

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Abstract

Parasitic nematodes infect over 1.5 billion people worldwide and are a burden on the agricultural and veterinary industries. Widespread use of the few available classes of anthelmintics have led to the development of anthelmintic resistance amongst these parasitic helminths. It is imperative that we find new therapeutics to address the looming threat of anthelminthic resistance. One method for developing new chemotherapies is through rational drug design. Using *C. elegans* as a surrogate for parasitic nematodes, we have taken the first steps to identify and characterize a small group of putatively druggable "nematode-specific" proteins by generating null mutants and endogenously-tagged reporters using CRISPR-Cas9 gene-editing technology. To our knowledge, this work is the first to define a family of novel manganese-selective cation diffusion facilitators in *C. elegans*. Although these are not essential proteins and are unlikely to be good drug targets, their newly discovered role in manganese tolerance advances our knowledge of the mechanisms that underlie manganese biology and toxicity.

Lay Summary

Parasitic worms are a diverse group of animals that infect plants, animals, and humans. Over 1.5 billion people around the world are infected with different types of parasitic worms, but there are only a few drugs to treat these infections. These drugs have become less effective over time as we continue to use them. We need to make new drugs to keep up with this 'arms race' against parasitic worms.

Proteins are essential building blocks of life. Many drugs work by attaching onto important proteins to alter their function. By identifying critical proteins in the worm, we can begin to design drugs that destroy the proteins' function to kill parasitic worms. We studied a small group of proteins to see whether they would be good targets for drugs against parasitic worms. We discovered that these proteins are important for the movement of manganese—an essential metal required to sustain life.

Preface

This project was completed in the Moerman Lab at the University of British Columbia under the counsel and supervision of Dr. Donald G. Moerman. I conceived and designed the project with guidance from Dr. Moerman and committee members, Dr. Abby Collier and Dr. Kota Mizumoto. Identification and characterization of manganese-selective cation diffusion facilitators (CDF) was done in collaboration with Dr. Ciro Cubillas at the Kornfeld Lab (Washington University, St. Louis, U.S.A.). The CDFs were identified as putative manganese-selective transporters in a previously published phylogeny by Dr. Cubillas (Cubillas *et al.* 2013). Dr. Cubillas developed and conducted the manganese-sensitivity assays with CDF null mutants created within the Moerman Lab. I designed and constructed the CRISPR knock-out strains for *ZK185.5*, *K07G5.5*, *R02F11.3*, *F41C6.7*, and *F56C9.3* (with help from technicians, Kiana Martin and Claudia Doell, for the latter two genes). To determine the developmental impact of manganese on *PDB1.1*(*ok3114*) and *R02F11.3* (*gk5490*) null mutants, I conducted manganese sensitivity experiments and imaged the *PDB1.1* null mutants using differential interference (DIC) contrast microscopy. I designed, constructed, and imaged all transcriptional and endogenously-tagged translational reporters of CDFs. Cathy Yan, an undergraduate student, was instrumental in the construct of *ZK185.5* transcriptional reporter. I also conducted all genetic crosses, brood-sizing, bioinformatic analysis, and data analysis for this thesis project.

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List of Abbreviations

ANOVA	Analysis of variance
BDM	2,3-butanedione monoxime
C. elegans	*Caenorhabditis elegans*
Cas9	CRISPR associated protein 9
CDF	Cation diffusion facilitators
CDS	Coding sequences
CGC	*Caenorhabditis* Genetics Centre
CRISPR	Clustered regularly interspaced short palindromic repeats
DIC	Differential interference contrast
DIOPT	DRSC Integrative Ortholog Prediction Tool
DRSC	*Drosophila* RNAi Screening Center
FeCH	Ferrochelatase
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
HGT	Horizontal gene transfer
IDT	Integrated DNA technologies
L1, L2, L3, L4	Larval stage 1, 2, 3, 4
LF	Lymphatic filariasis
LMIC	Low and middle-income countries
MDA	Mass drug administration
NaOH	Sodium hydroxide
NAMM	Noble agar minimal media

NGM	Nematode growth medium
Nramp	Natural resistance-associated macrophage proteins
PCR	Polymerase chain reaction
RFP	Red fluorescent protein
RNAi	RNA interference
RNP	Ribonucleoprotein
IDT	Integrated DNA Technologies
SLC	Solute carrier
tracrRNA	Trans-activating CRISPR RNA
WBP	WormBase ParaSite
WHO	World Health Organization

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I would like to extend my thanks and appreciation to the Mizumoto Lab for gifting pSM GFPnovo2, particularly Ardalan Hendi for all his help and advice on how to construct a transcriptional reporter, and Mizuki Kurashina for his patience in teaching me how to use the confocal microscope.

My heartfelt gratitude to all the members of the Moerman Lab, past and present, whom I consider my lab family. Special thanks to Mark Edgley for his wisdom and sage advice about life both inside and out of the lab; Dr. Stephane Flibotte for his advice on data analysis and teaching me basic UNIX, Erica Li-Leger for teaching me DIC microscopy and being a supportive friend; Lisa Fernando for providing all the plates and plastics that enabled me to do this work, and Kiana Martin, Viktorija Juciūtė, and Claudia Doell for their help, uplifting presence and encouragement. I have been graced by the people in this lab who have brought so much light, joy, and laughter into my life.

I would like to express my thanks to the local worm community for their camaraderie and generosity. In particular, I would like to thank Dr. Troy McDiarmid for both his friendship and sharing his contagious excitement for science and collaboration, and Ethan Fortes for his frequent small acts of kindness.

I would like to acknowledge the UBC Cell Graduate Program, NSERC, and CIHR for their financial support of this work.

Most of all, I'd like to thank my friends, family, and partner, Siim Karu, for their unyielding love and support.

Dedication

I dedicate this to 爺爺. The best parts of me are because of you.

Chapter 1: Introduction

1.1 Helminthiases: entering the neglected tropical disease wormhole

Helminths are parasitic worms that span two phyla: Nematoda (roundworms) and Platyhelminthes (flatworms). They are diverse and ubiquitous organisms that devastate the health and productivity of crops and livestock, threatening the food security and economies of many countries (Charlier *et al.* 2014; Coyne *et al.* 2018). Helminths are also among the most common infectious agents to afflict people and have plagued society since time immemorial. It is estimated that approximately 1.5 billion people are infected by helminths (Table 1.1, Pullan *et al.* 2014). Most infections caused by these parasitic worms are considered neglected tropical diseases due to their prevalence in tropical, and low and middle-income countries (LMIC) (Hotez *et al.* 2008). The burden of illness and disability in LMICs keep them in an unfortunate cycle of poverty (Hotez *et al.* 2008; Makenga Bof *et al.* 2019). However, helminth infections are not unique to impoverished nations; they also highlight the gross inequalities that exist even within the wealthiest of nations. For example, hookworms were once thought to have been nearly eradicated in the American South (Bleakley 2007), but one study conducted in rural Lowndes County, Alabama, U.S.A. found over a third of residents had contracted hookworms (*Necator americanus*) due to poor sewage management (McKenna *et al.* 2017). Today, as the COVID-19 pandemic continues, patients that are infected with *Strongyloides stercoralis* are precluded from dexamethasone (an immunosuppressant) treatment for COVID-19 (Marchese *et al.* 2020; Shirley and Moonah 2021; De Wilton *et al.* 2021) due to the risk of fatal Strongyloides Hyperinfection Syndrome (Kassalik and Mönkemüller 2011). Although often regarded as transient and easily treatable in high-income countries, helminth diseases can cause a host of problems when patients face severe or chronic infections—a reality that's especially common in LMICs.

Route of infection	Disease	Infectious agent(s)	Estimated # of people affected globally
Soil-transmitted helminths	Ascariasis	*Ascaris lumbricoides*	819.0 million[1]
	Hookworm disease	Hookworms (i.e. *Necator americanus*, *Ancylostoma duodenale*)	438.9 million[1]
	Trichuriasis	*Trichuris trichiura*	464.6 million[1]
Vector-borne helminths	Schistosomiasis	Blood flukes (*Schistosoma* spp.)	252 million[2]
	Lymphatic filariasis	*Wuchereria bancrofti*, *Brugia malayi*, *Brugia timori*	38.5 million[2]
	Onchocerciasis	*Onchocerca volvolus*	15.5 million[2]
	Loiasis	*Loa loa*	13.0 million[3]

[1,2,3] Estimates gathered from Pullan *et al.* (2014) [1], Vos *et al.* (2016) [2], and Hotez *et al.* (2008)[3]

Table 1.1 Global prevalence of major helminthiases. Individuals can be infected with more than one parasitic helminth at the same time.

1.1.1 Mass drug administration and the emergence of anthelminthic resistance

Many communities on the continents of Asia, Africa, and Latin America have long grappled with the problem of helminthiases (Hotez *et al.* 2008). International initiatives such as the Global Program to Eliminate Lymphatic Filariasis (Manyeh *et al.* 2020) and the London Declaration on Neglected Tropical Disease (Vercruysse *et al.* 2012) have sought to eliminate helminth infections around the world through mass drug administration (MDA) programs. However, eliminating helminthiases endemic to LMICs pose special challenges: the remoteness of communities, affordability, and pervasive distrust of institutions (Ames *et al.* 2019). These circumstances demand drugs to be more than just effective—they must be stable enough to transport in a variety of conditions, cheap enough to produce at scale, potent enough to be effective in a single dose, and safe enough to administer to millions. With the assistance of large pharmaceutical companies and generic drug producers, the World Health Organization (WHO) is able to distribute such preventative chemotherapies to over a billion people worldwide (WHO 2020). However, only a few classes of anthelminthic drugs have been approved for the treatment of human-parasitizing helminths. Widespread administration of these limited drug classes

threaten the efficacy and longevity of their use (Table 1.2). Additionally, current treatments can also cause severe adverse effects in patients with high microfilarial loads (such is the case for *Loa loa* infections), which complicate or preclude mass drug administration programs in places where loiasis is endemic to the region (Herrick 2019; Makenga Bof *et al.* 2019). Moreover, no new anthelminthic drug has been developed for the treatment of human helminthiases for over 30 years (Kaplan and Vidyashankar 2012). In veterinary settings, resistance to new anthelminthic drugs appear only within a couple of years of its use (Scott *et al.* 2013; Wit *et al.* 2021). Fortunately, despite the many years of MDAs, anthelminthic resistance has not been widely reported in human helminthiases (Wit *et al.* 2021). Although the current MDA approach has been largely effective, evidence from agriculture, veterinary medicine, and clinical cases suggest that anthelmintic resistance still looms on the horizon. Studies have already shown evidence of anthelminthic resistance in human-parasitizing helminths (Krücken *et al.* 2017; Furtado *et al.* 2019; Orr *et al.* 2019) and of the waning efficacy of benzimidazoles (De Clercq et al 1997; Albonico 2003; Flohr *et al.* 2007) and pyrantel (Reynoldson *et al.* 1997); all may be indications of the impending hurdles to treatment ahead. To provide more diverse treatment options for helminth infections, we have to better understand helminth biology in the context of drug development.

Drug class	Drug(s)[+]	Use case(s)[±]	Mode of action	Reference
Piperazines	diethylcarbamazine	Used to treat LF	Unknown	
Benzimidazoles	albendazole, mebendazole, triclabendazole	Used to treat soil-transmitted helmith infections (e.g. hookworms, ascariasis, trichuriasis) and LF	Binds to ß-tubulin and prevents further polymerization of microtubules	Lacey 1990 (review)
Macrocyclic lactones	ivermectin, moxidectin	Used to treat LF and onchoceriasis	GABA and glutamate-gated chloride channel agonist	Dent *et al.* 2000
Tetrahydroisoquinolines	praziquantel	Used to treat schistosomiasis	Unknown	

[+]Drug class information gathered from Drugbank (go.drugbank.com, Wishard *et al.* 2018)
[±]Use case information from WHO (2018), Montresor *et al.* 2020, and Sepúlveda-Crespo *et al.* 2020

Table 1.2 Anthelminthics used in mass drug administration programs for the prevention and elimination of helminthiases.

As there are no surveillance systems in place to actively monitor the development of anthelmintic resistance in MDAs programs (Vlaminck *et al.* 2018) and no coordinated plan to address resistance should it arise (Vercruysse *et al.* 2012), we have to work diligently to find other treatments. Informed and cost-effective anthelminthic drug-discovery schemes must be devised to counter the global burden of helminthiases and curb the rise of anthelminthic resistance.

1.2 The rationale behind rational drug design for combating helminthiases

There are two major approaches to drug development: 1. high through-put screens of large compound libraries, and 2. rational drug design. Typically, high through-put screens require a lot of resources to rapidly sift through thousands of compounds before identifying a few that elicit the phenotype for which you're screening for. There are several recent examples of successful high-throughput screens for anthelmintic compounds (examples include Kaminsky *et al.* 2008, Burns *et al.* 2015, and Mathew *et al.* 2016). Alternatively, rational drug discovery offers a different approach that starts by identifying a protein target of interest and develops or

screens for ligands that bind the target. To our knowledge, no anthelminthic drug on market has been developed in such a way, but it has been widely recognized by the parasitic helminth community and the WHO as a viable approach to address the development of anthelminthic resistance (Kumar *et al.* 2007, WHO 2012, Wever *et al.* 2015, Coghlan *et al.* 2018). Overall, rational drug design may be better at reducing off-target effects and recovering more relevant drugs as the drugs are intentionally designed or selected to be target-selective whereas large-scale screening may yield compounds that affect multiple proteins.

A suitable drug target for rational drug design must fulfill two critical requirements: 1. it is predicted to be "druggable" and 2. perturbation of its activity results in a desired phenotype. A druggable protein is one that has membership in a protein family commonly targeted by existing drugs (e.g. kinases, ion channels, and G-protein coupled receptors) and, by proxy, is also likely to bind to other chemical compounds (Hopkins and Groom 2002). In a post-genomic era, we are able to probe entire genomes to identify novel drug targets. In 2007, Kumar *et al.* were able to conduct a genome-wide search for essential and druggable proteins in the first published helminth genome, *Brugia malayi*. Since then, due to the burgeoning growth and increasing affordability of whole-genome sequencing, over 130 de novo helminth genomes have since been assembled (Howe *et al.* 2017). This boon in genome technology has spurred the development of WormBase's sub-portal: WormBase ParaSite (WBP). WBP is a comprehensive database of helminth genomes and comparative genomics tools for free-living and parasitic worms (Howe *et al.* 2016, Howe *et al.* 2017); consequently, it's a useful resource for identifying putatively druggable proteins that are present across numerous helminth species.

One emerging class of drug targets are the lesser-known solute carriers (SLCs), a large and diverse superfamily of transmembrane transporters that move a variety of solutes including

amino acids, neurotransmitters, metabolites, and metals (Rives *et al.* 2017, Zhang *et al.* 2019). They are the second largest class of transmembrane proteins (next to GPCRs) and are drug targets for the treatment of depression, diabetes, cancer, and inflammatory diseases (Rives *et al.* 2017), but there are relatively few drugs that target SLCs and they still remain a large "untapped potential" for drug development. It is precisely because of their ability to be targeted by small molecules, their importance for organismal health, and their diversity of functions, that our study has chosen to characterize a subset of SLCs (specifically SLC30s, also known as cation diffusion facilitators) as potential anthelminthic drug targets.

For anthelminthic discovery, we must also provide evidence of a protein's functional importance in parasites by either agonizing or antagonizing the putative drug target (McVeigh *et al.* 2012, Wever *et al.* 2015). At this time, this cannot be done in parasitic worms as we lack the tools necessary to conduct such an experiment, therefore it was completed in a genetically tractable *C. elegans* model.

1.3 *C. elegans* as a surrogate for parasitic nematodes

Nematodes are major contributors to global helminthiases. The Nematoda phylum is split into five different clades (Clades I-V) based on the sequences of small subunit ribosomal DNA; and parasitic nematodes can be found in every clade (Blaxter *et al.* 1998). Within Clade V are members of the *Caenorhabditis* genus, as well as important parasitic helminths such as *Haemonchus contortus*, *Necator americanus*, and *Anclyostoma duodenale* (Blaxter *et al.* 1998; Holden-Dye and Walker 2012). *Caenorhabditis elegans* is a small free-living nematode that is amenable to a variety of experimental techniques due to its short life-cycle, large brood size, modestly-sized and fully-sequenced genome, and ease of maintenance (Sepulveda-Crespo *et al.* 2020; Wit *et al.* 2021). Recognizing parasitic nematodes have complex life-histories that would

make rearing costly and time-consuming, Simpkin and Coles (1981) were early proponents of using *C. elegans* as a surrogate for parasitic worms in anthelminthic screens because of their sensitivity to a range of existing anthelmintics including benzimidazoles, avermectin, and levamisole. In their seminal paper, they also suggest that it should be possible to elucidate anthelminthic mechanisms of action and modes of resistance using *C. elegans*; and indeed, it has been a useful tool for both the discovery and study of anthelminthics over the many years (Wit *et al.* 2021).

The use of *C. elegans* in high-throughput drug screens has yielded several candidate compounds (Kwok *et al.* 2006; Burns *et al.* 2012; Burns *et al.* 2015; Mathew *et al.* 2016) and a *Bacillus thuringiensis* (Bt) toxin (Marroquin *et al.* 2000) for anthelminthic development. In two recent studies conducted by us and another research group, we both employed this method of drug discovery and were able to identify similar nematicidal compounds (Burns *et al.* 2015; Mathew *et al.* 2016). Both research groups were also able to identify the target MEV-1 protein subunit within mitochondrial complex II, define its binding pocket, and elucidate the possible mechanisms of resistance using the many genetic tools available to *C. elegans* researchers (Burns *et al.* 2015; Mathew *et al.* 2016). The newly discovered compounds were then cross-validated in parasitic nematodes: *Cooperia oncophora*, *Haemonchus contortus* (Burns *et al.* 2015), and *Meloidogyne hapla* (Mathew *et al.* 2016). These two examples successfully demonstrate the usefulness of *C. elegans* as a surrogate for parasitic nematodes in anthelminthic drug discovery.

Previous studies have also used *C. elegans* as a surrogate for the identification and validation of anthelminthic drug targets (Kumar *et al.* 2007; Wever *et al.* 2015). This is possible owing to the many orthologs and biological processes shared between *C. elegans* and parasitic nematodes. There are many examples (Kwa *et al.* 1995; Britton and Murray 2002; Kampkotter *et*

al. 2003; Couthieret *et al.* 2004; Gava *et al.* 2014; Wheeler *et al.* 2020) of phenotypic rescue through heterologous expression of parasite genes in *C. elegans* to support (though not confirm) orthology predictions between free-living and parasitic worms (Gilleard 2004; Holden-Dye and Walker 2012). In 2015, Wever, Farrington, and Dent also used *C. elegans* to validate nematode-specific acetylcholine-gated chloride channels as potential targets for anthelminthic drug agonists. Given that few molecular tools are available for the characterization of gene function in helminths, parasitic nematologists leverage the rich database of functional annotations in the *C. elegans* genome to help infer gene function in other nematode genomes (Holden-Dye and Walker 2012; Fernández-Breis et al 2016). Furthermore, a subset of these orthologs are predicted to be nematode-specific (Parkinson *et al.* 2004), which is an important attribute for a good nematicidal drug target as it reduces the risk of off-target effects (Santos *et al.* 2017). Even within large protein families commonly found in many domains of life, such as the nicotinic acetylcholine receptor (nAChR) family, there are still members that are specific only to nematodes and can be exploited as drug targets (Holden-Dye and Walker 2012), as is the case with Monepantel (Kaminsky *et al.* 2008, Kaminsky and Rufener 2012). As part of this thesis work, we characterized a small family of cation diffusion facilitators (CDFs) in *C. elegans* that are orthologous across many parasitic helminths (both nematodes and platyhelminths), free-living *Caenorhabditis* species, as well as fungi and plants, but excluded from humans and livestock, to investigate whether they would be suitable drug targets for anthelmintic development.

1.3.1 Cation Diffusion Facilitators

Cation diffusion facilitators are members of the solute carrier superfamily that transport cations out (though not exclusively) of the cytoplasmic space and into organelles or the

extracellular domain (Kolaj-Robin *et al.* 2015). CDFs are present across every domain of life (Montanini *et al.* 2007; Cubillas *et al.* 2013) and play a critical role in metal homeostasis (Kolaj-Robin *et al.* 2015) by protecting against metal toxicity (Davis *et al.* 2009; Roh *et al.* 2013) and regulating cellular processes in growth and development (Bruinsma *et al.* 2002; Yoder *et al.* 2004). For most organisms, it is estimated that metals interact with more than a third of all proteins, playing vital roles in protein function and cell signaling (Hood and Skaar 2013; Buccella *et al.* 2019). Studies of CDF mutants have determined that some CDF family members are required for the survival of progeny in mice (Huang and Gitschier 1997), protection against neurotoxicity in humans (Anagianni and Tuschl 2019), and even virulence in pathogenic bacteria (Grunenwald *et al.* 2019). Evidence that disrupted CDF function can lead to poor survival outcomes in different organisms indicate that CDFs could potentially be suitable drug targets for anthelmintic development.

Both an abundance and a lack of metal ions can lead to deleterious effects in all organisms; as such, careful control of metal concentrations is necessary for all life. This thesis work focuses on a small family of paralogous CDFs that had not been previously characterized and were annotated as zinc efflux transporters. Our study, in collaboration with Dr. Ciro Cubillas from the Kornfeld Lab, revealed that these proteins are, in actuality, manganese-selective CDFs—the first described in *C. elegans*.

1.3.2 Disorders of manganese dyshomeostasis

Manganese is an essential trace metal involved in a variety of biological processes including innate immunity (Haase 2018), development and metabolism (Avila *et al.* 2013), and is likely the most well-known as a co-factor of Manganese Superoxide Dismutase (Mn-SOD) which buffers against oxidative stress in the mitochondria (Holley *et al.* 2011). Disruption of

normal manganese homeostasis have been shown to cause long-term neurological damage in humans (Avila *et al.* 2013; Wahlberg *et al.* 2018). The neurotoxicity caused by excess manganese, also called "manganism", presents similarly to Parkinson's disease (Horning *et al.* 2015). Manganism has multiple etiologies, but they can be broadly split into two types: environmental and genetic. Environmental manganese toxicity was initially described in 1837 by J. Couper, and is often caused by industrial pollutants or air contamination in environments where mining and welding take place (Racette 2014). Hereditary manganism was described much more recently in 2012 by two independent research groups that identified mutations in SLC30A10, a manganese CDF, as the underlying cause (Tuschl *et al.* 2012; Quadri *et al.* 2012).

To study the molecular underpinnings of manganese toxicity, particularly in neurons, *C. elegans* has been used as a model to understand the mechanisms behind manganism. Studies have demonstrated that excess manganese in *C. elegans* lead to decreased lifespan, stunted growth or development (Xiao *et al.* 2009; Angeli at al. 2014), and damaged dopaminergic neurons (Benedetto *et al.* 2010; Settivari *et al.* 2009). However, no manganese-selective efflux transporters (i.e. Mn-CDFs) have been identified in *C. elegans* and studies have relied solely on the expression of human SLC30A10 from an extrachromosomal array in *C. elegans* (Chen *et al.* 2015b; Leyva-Illades *et al.* 2014). The discovery of a nematode and manganese-selective transporter may allow us to further investigate how manganese is regulated in *C. elegans* and possibly in other domains of life as well.

1.4 Summary

Despite the immense burden that helminthiases place on our global community, the amount of resources dedicated to the treatment and eradication of these diseases is sorely inadequate given the scale of the problem. Their enormous impact on human health compels us

to take action to eliminate these parasites through research and development of more pharmaceutical interventions. The growth and application of whole-genome sequencing technology in helminths has allowed us to identify potential drug targets that are orthologous across parasitic nematodes, platyhelminths, and the free-living *Caenorhabditis* species. We chose to focus our efforts on the characterization of a small family of previously uncharacterized CDFs. Using *C. elegans* as a model for parasitic helminths, we are able to use sophisticated genetic and molecular tools to study these metal transporters. Although these proteins may not be ideal targets for anthelminthic development, their newly uncovered identity as manganese-selective efflux transporters offers insights into the world of manganese biology and the evolutionary history of CDFs.

Chapter 2: Methods

2.1 Bioinformatic search of druggable orthologs

WormBase Parasite (WBP) BioMart (www.parasite.wormbase.org/biomart) was used to mine predetermined orthologs predicted by Ensembl Compara. We queried for orthologs that are present between *C. elegans* and ten other nematode species (*Caenorhabditis briggsae*, *Caenorhabditis remanei*, *Strongyloides ratti*, *Meloidogyne hapla*, *Meloidogyne incognita*, *Haemonchus contortus*, *Loa loa*, *Onchocerca volvulus*, *Brugia malayi*, and *Ascaris suum)*, while excluding orthologs present in humans or any of the following five model organisms: *Drosophila melanogaster*, *Mus musculus*, *Rattus norvegis*, *Danio rerio*, and *Saccharomyces cerevisiae*. Putatively druggable orthologs were determined by filtering associated gene ontology terms and InterPro ID annotations for protein families that are considered common drug targets (Hopkins and Groom 2002, Coghlan *et al.* 2018). These putatively druggable orthologs were then further scrutinized using DIOPT, a tool that integrates multiple orthology prediction programs (Hu et al 2011), to eliminate candidates that may have orthologs in our outgroup species as predicted by other algorithms. Finally, these putatively druggable and de facto "nematode-specific" orthologs were curated and selected for characterization based on additional available information such as expression data, RNAi and mutant phenotypes, and existing publications.

2.2 Generation of CRISPR-Cas9 deletion and translational reporter strains

Deletion mutants for *ZK185.5*, *K07G5.5*, *F41C6.7*, *F56C9.3*, and *R07F11.3* and endogenously-tagged C-terminal fluorescent reporters for *PDB1.1*, *ZK185.5*, and *F41C6.7* were generated using a CRISPR-Cas9 protocol developed by Norris *et al.* (2015) and optimized for high throughput by Au *et al.* (2018).

The deletion dual-marker selection cassette contains G418 drug resistance and with either pharyngeal (*loxP* + P*myo-2::GFP::unc-54* 3' UTR + P*rps-27::neoR::unc-54* 3' UTR + *loxP*) or body-wall (*loxP* + P*myo-3::GFP::unc-54* 3' UTR + P*rps-27::neoR::unc-54* 3' UTR + *loxP*) GFP. The fluorescent-protein tag dual-marker selection cassettes maintain the same selection cassette as the deletion vector but is flanked by two halves of either GFP or RFP. The suite of dual-marker selection cassettes was graciously gifted by Dr. John Calarco (University of Toronto, Canada). CRISPR guide RNAs (crRNA) were designed using the *C. elegans*-specific CRISPR guide RNA selection tool (www.genome.sfu.ca/crispr) and synthesized by Integrated DNA Technologies (IDT). Homology arms of approximately 450-bp (gBlocks synthesized by IDT) were assembled into the dual-selection cassette plasmid using NEBuilder Hifi DNA Assembly Kit (New England Biolabs).

The crRNAs (IDT) were independently duplexed with tracrRNA (IDT), then complexed with Cas9 protein (gifted by Dr. Geraldine Seydoux, John Hopkins University, Baltimore, U.S.A.) into a ribonucleoprotein (RNP) complex as outlined in IDT's CRISPR protocol. A mix of 0.5 µM RNP complex, 50 ng/µl repair template, 5 ng/µl pCFJ104 (*Pmyo*-3::mCherry) and 2.5 ng/µl pCFJ90 (P*myo-2*::mCherry) co-injection markers were injected into the gonads of PD1074 or VC3504 (both are derivatives of the same N2 wildtype strain) young adult worms on the same day of assembly. Injected worms were distributed 4 per 60 mm NGM plate streaked with OP50 *E. coli*. The plates were drugged with 25 mg/mL G418 the following day. After 5 days, F_1 worms were screened for putative integrants that have dim and even expression of either pharyngeal or body-wall GFP without any RFP. Putative integrants are validated by PCR as outlined in Au *et al.* (2018). CRISPR deletion strains were backcrossed to N2 at least 5 times. Dual-marker selection cassettes were excised from fluorescent-protein tagged strains by injecting

pDD104 (P*eft-3*::Cre, gifted by Dr. John Calarco, University of Toronto, Canada) at 50 ng/µl concentration with pCFJ90 (P*myo-2*::mCherry), incubating F_1 progeny at 25˚C for two to three days, and screening for animals that have lost the dual-marker cassette. All strains were validated again by PCR at the selection cassette insertion junctions as described by Au *et al.* (2018).

2.3 Generation of multi-gene deletion strains using genetic crosses

Single CDF CRISPR deletion mutant were crossed together to create double or triple CDF deletion mutant strains. Male worms were obtained by incubating CDF mutant L4 hermaphrodites in 10% ethanol for 30 minutes. The hermaphrodites were then removed from the ethanol solution and placed onto a 60 mm NGM plate streaked with OP50. Male progeny were selected and used to propagate a male line of the mutant strain. Mating crosses were set up between different CDF strains. Cross progeny could be distinguished by the presence of multiple fluorescent markers (i.e. both pharyngeal and body-wall GFP) or relative brightness to parental strains (i.e. cross-progeny have brighter GFP expression than either parental strains). All multi-gene deletion strains were validated by PCR at selection cassette insertion junctions and assayed for wild-type copies of the genes as described by Au *et al.* (2018).

2.4 Generation of CDF deletion strains with GFP-marked dopaminergic neurons

CDF deletion mutants were crossed with a strain containing GFP-marked dopaminergic neurons (P*dat-1*::GFP), BZ555 (*egIs1*) (Davies *et al.* 2003). Males worms were created using the method outlined above for BZ555 or the CDF deletion mutant. Heterozygous cross progeny were obtained from crosses and allowed to self-fertilize for one generation. Homozygous F2 progeny were selected for using the presence of both P*dat-1*::GFP expression and bright CRISPR selection marker (either *Pmyo-2*::GFP or *Pmyo-3*::GFP) and validated by PCR. The dual-

selection marker was removed through the introduction of Cre recombinase as outlined in Norris *et al.* (2015).

2.5 Generation of transcriptional reporter of ZK185.5

The transcriptional reporter of *ZK185.5* was created in a pSM GFPnovo2 plasmid backbone (gifted by Dr. Kota Mizumoto, University of British Columbia, Vancouver, Canada). A 5-kb region upstream of *ZK185.5* was PCR amplified with Phusion Hi-Fidelity DNA polymerase (Thermo Fisher Scientific) from a WRM068dC03 fosmid template (Perkins 2011) to capture the promoter region of *ZK185.5*. The promoter fragment was then cloned between *Sph*I and *Asc*I sites of pSM GFPnovo2 plasmid (Hendi and Mizumoto 2018). An injection mix of 45 ng/µL P*ZK185.5*::GFPnovo2, 5 ng/µl pCFJ104 (*Pmyo*-3::mCherry), and 2.5 ng/µl pCFJ90 (P*myo-2*::mCherry) were injected into the gonads of young adult PD1074 wildtype worms (Mello et al. 1991).

2.6 Brood sizing

Brood sizes of CDF deletion mutants were assayed by plating a single L4 worms onto 60 mm agar plates with OP50 and kept at 20˚C. P_o worms were allowed to lay eggs for a 24-hour period before being transferred to a new plate. Each P_o was transferred to a new plate four times (5 plates total). F_1 progeny were counted and aspirated away 48-hours after removal of the P_o. Plates were also inspected for any remaining F_1 progeny at the 72-hour mark after removal of the P_o worm. Statistical significance was calculated with one-way ANOVA using R.

2.7 Metal sensitivity assays

Metal sensitivity assays were conducted by our collaborator, Dr. Ciro Cubillas (Kornfeld Lab, Washington University, St. Louis, U.S.A.). Worms were age synchronized by lysing gravid adults with a sodium hydroxide and bleach solution and allowing the eggs to hatch overnight and

arrest as L1 larvae in M9 solution. The L1 animals were then grown on noble agar minimal media (NAMM) plates (Bruinsma *et al.*, 2008) supplemented with zinc sulfate ($ZnSO_4$), copper chloride ($CuCl_2$), or manganese chloride tetrahydrate ($MnCl_2$) at the desired concentrations and seeded with concentrated OP50. After 3 days, animals were washed twice with M9 solution, paralyzed with 25mM tetramisole hydrochloride in M9 solution, and mounted on a 2% agarose pad on a microscope slide. Images were captured with a Zeiss Axioplan 2 microscope equipped with a Zeiss AxioCam MR digital camera. The length of individual animals was measured using ImageJ software (Schneider *et al.* 2012) by drawing a line from the nose to the tail tip.

Recovery from manganese challenge was conducted at the Moerman Lab on 500 µM Mn plates using the same protocol as described above. Approximately 20 to 30 P_os were removed from the high manganese environment at roughly the 24- and 48-hour mark and placed onto standard NGM plates with OP50 to watch for recovery.

2.8 Microscopy

Images of transcriptional and translational reporters were taken using a Zeiss LSM800 Airyscan confocal microscope (Carl Zeiss, Germany). L4 stage worms were mounted and immobilized in a mixture of 0.225 M 2,3-butanedione monoxime (BDM; Sigma-Aldrich, U.S.A.) and 25 mM levamisole (Sigma-Aldrich, U.S.A.) solution on 2% agarose pads. Images were captured and analysed using Zeiss ZEN Imaging Software (Blue edition). Translational reporters were imaged at 63x magnification under an oil immersion lens. Transcriptional reporter expression of *ZK185.5* was imaged at 5x magnification.

Mutant *PDB1*.1 (*ok3114*) and *R02F11.3* (*gk5490*) worms were imaged after 3 days on 500 µM Mn NAMM plates using a Zeiss Axioplan 2 microscope with differential interference

contrast (DIC) optics with Zeiss ZEN 2.6 imaging software (Carl Zeiss, Germany). Worms were immobilized and mounted in the same method as described above.

Chapter 3: Results

3.1 There are thirty-three putative druggable "nematode-specific" orthologs

An ideal anthelmintic has a specific-enough target to effectually eliminate or minimize adverse effects on the host. We used WormBase ParaSite (WBP) BioMart (Smedley *et al.* 2009) to identify "nematode-specific" putative drug targets for further characterization in *C. elegans*. WBP BioMart is a unique and powerful tool that allowed us to query the collection of 137 nematode and platyhelminth genomes hosted at WBP (Version 13.0; Howe *et al.* 2016; Howe *et al.* 2017). This publicly available data mining tool uses Ensembl Compara to identify orthologs shared between our nematodes of interest while simultaneously eliminating those found in our outgroup. Specifically, we were interested in orthologs found across 11 nematode species: *Caenorhabditis elegans* (free-living model nematode*), Caenorhabditis briggsae* (free-living nematode), *Caenorhabditis remanei* (free-living nematode), *Strongyloides ratti* (rodent model parasite), *Meloidogyne hapla* (plant parasite), *Meloidogyne incognita* (plant parasite), *Haemonchus contortus* (sheep parasite), *Loa loa* (human parasite), *Onchocerca volvulus* (human parasite), *Brugia malayi* (human parasite), and *Ascaris suum* (a pig parasite which is also closely related to the human parasite, *Ascaris lumbricoides*). In addition, we chose to exclude orthologs present in humans or any of five model organisms: *Drosophila melanogaster*, *Mus musculus*, *Rattus norvegis*, *Danio rerio*, and *Saccharomyces cerevisiae*. Presently, we have identified 373 "nematode-specific" orthologs with their associated gene ontology terms and protein domain annotations.

Using gene ontology terms and InterPro protein domain annotations, we filtered our list of "nematode-specific" orthologs substantially to only include proteins that may be considered 'druggable' (i.e. fall within families of proteins commonly targeted by existing drugs, see

Appendix A.1). Druggable proteins fall within protein families that are often targeted by therapeutic compounds. Kinases, GPCRs, and ion channels represent roughly 40% of known pharmaceutical targets, making these the top three most druggable protein families (Hopkins and Groom 2002; Santos *et al.* 2017). Over 120 gene families make up the remaining pharmacological space (Hopkins and Groom 2002). However, this "guilt by association" approach does not guarantee that any particular protein would be suitable as a drug target. Even so, this is often one of the first measures taken to identify candidate proteins for further characterization in rational drug design (Coghlan *et al.* 2018; Wever *et al.* 2015; Agüero *et al.* 2008). Using both gene ontology terms and InterPro ID, we identified 33 proteins that fall within the top three druggable families: GPCRs, kinases, and ion channels (Santos *et al.* 2017), as well as transmembrane transporters (Figure 3.1).

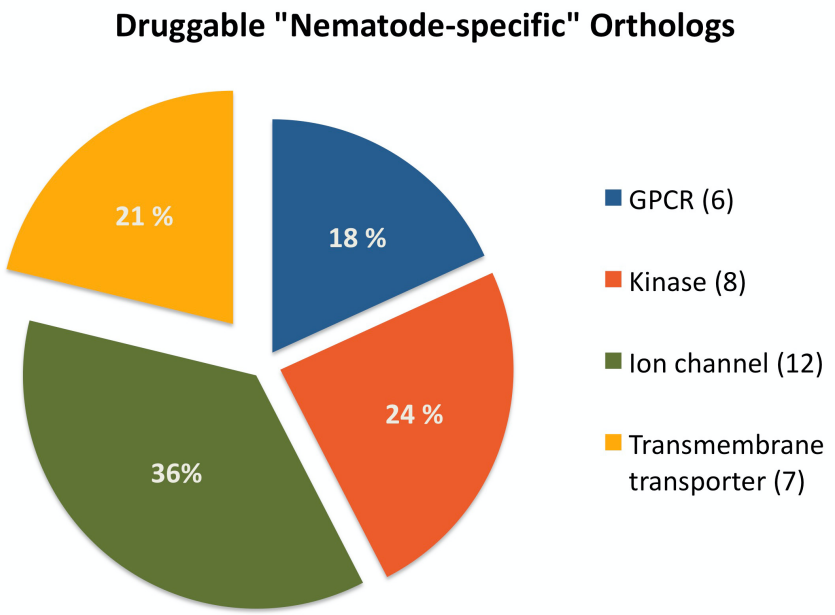

Figure 3.1 There are 33 'nematode-specific' druggable orthologs. We identified 33 'nematode-specific' orthologs that fall within four major druggable protein families: GPCRs (6 proteins), kinases (8 proteins), ion channels (12 proteins), and transmembrane transporters (7 proteins).

Of the four druggable protein families, transmembrane transporters were of particular interest as it includes two major classes of proteins implicated in disease and drug delivery/metabolism: ATP-binding cassette (ABC) transporters and solute carriers (SLCs) (Russel 2010; Tarling *et al.* 2013; Štefková *et al.* 2004). SLCs are an emerging class of drug targets comprised of proteins that transport various substrates across membranes and are implicated in the development of several human diseases (Lin *et al.* 2015). SLCs move a large variety of essential molecules including amino acids, vitamins, glucose, and trace metals, but they also play a role in drug absorption, elimination, and distribution (Russell 2010; Rives *et al.* 2017; Nigam 2015). Mutations in over 80 SLCs have been implicated in Mendelian disorders such as Amish lethal microcephaly (Lin *et al.* 2015), cystinuria (Schaller and Lauschke 2019), and familial manganism (Quadri *et al.* 2012; Tuschl *et al.* 2012). Some SLCs have also been associated with more common disorders with multiple etiologies such as Type 2 diabetes, inflammatory bowel disease, and cancer (Lin *et al.* 2015; Schumann *et al.* 2020; Bai, Moraes, and Reithmeier 2017). Despite the importance, abundance, and druggability of the diverse SLC superfamily of transporters, they remain a relatively understudied group of drug targets (César-Razquin *et al.* 2015; Lin *et al.* 2015; Rives *et al.* 2017; Wang *et al.* 2020).

From our short-list of "nematode-specific" proteins, we directed our attention to two paralogous SLC30s: *ZK185.5* and *R02F11.3*—neither of which have been previously characterized. SLC30 is a subfamily of solute carriers known as CDF, which specialize in the efflux of metal cations out of the cytosol and into organelles or extracellular space (Palmiter and Huang 2004). Phylogenetic analysis using TreeFam (Guindon *et al.* 2010; Ruan *et al.* 2008) show these two CDFs have four other paralogs (*K07G5.5*, *F41C6.7*, *PDB1.1*, and *F56C9.3*) in *C. elegans* (Figure 3.2)—all of them predicted to be "nematode-specific" with many having

orthologs in at least two parasitic nematodes of interest (Table 3.1). These six proteins were further scrutinized for potential human orthologs using DIOPT (Version 8; www.flyrnai.org/cgi-bin/DRSC_orthologs.pl), a platform that aggregates several orthology prediction algorithms (Hu *et al.* 2011). Only one of fifteen orthology prediction tools in DIOPT, eggNOG (Version 5.0), identified all six proteins as orthologs of human SLC30A9. However, each gene had less than 22% sequence identity with the human protein and all had a low DIOPT ortholog confidence score (Table 3.1); this gives further credence that these CDFs are likely "nematode-specific". Furthermore, four (*ZK185.5*, *K07G5.5*, *PDB1.1*, and *F41C6.7*) of the "nematode-specific" CDFs are expressed predominantly in the intestine (Hutter and Suh 2016; Cao *et al.* 2017) which makes these targets more accessible for potential drug interactions (Kaletta and Hengartner 2006). Taken altogether, we endeavored to characterize all six CDFs and determine whether they would be suitable candidates for drug development.

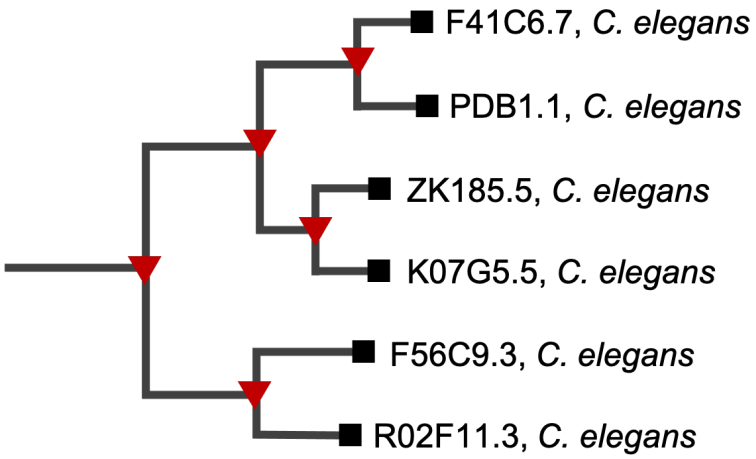

Figure 3.2 *C. elegans* has six paralogous cation diffusion facilitators. This phylogenetic relationship was retrieved from TreeFam database which creates a consensus tree from both nucleotide and protein-generated phylogenetic trees (Ruan *et al.* 2008; Vilella *et al.* 2009; Guindon *et al.* 2010), and is in agreement with PANTHER and WormBase-Compara (EnsEMBL-Compara) results.

CDS name	Predicted Human Ortholog	DIOPT Score	Rank	Prediction Derived From	Parasites with predicted orthologs†
F41C6.7	SLC30A9	1	low	eggNOG	*T. muris**
ZK185.5	SLC30A9	1	low	eggNOG	*B. malayi, S. ratti, O. volvulus, T. muris**
K07G5.5	SLC30A9	1	low	eggNOG	*B. malayi, S. ratti, O. volvulus, T. muris**
PDB1.1	SLC30A9	1	low	eggNOG	*T. muris**
R02F11.3	SLC30A9	1	low	eggNOG	*B. malayi, S. ratti, O. volvulus, T. muris**
F56C9.3	SLC30A9	1	low	eggNOG	*B. malayi, S. ratti, O. volvulus, T. muris**

† Parasite ortholog prediction from WormBase.
* *Trichuris muris* is a mouse parasite similar to the human parasite, *Trichuris trichiura*.

Table 3.1 Nematode cation diffusion facilitators are unlikely orthologs of human SLC30A9. DIOPT predicts low orthology with human SLC30A9 from eggNOG algorithm only.

3.2 Cation diffusion facilitators are non-essential proteins

To begin interrogating the function(s) of our CDFs of interest, we generated CRISPR knock-outs for five (*ZK185.5*, *K07G5.5*, *F41C6.7*, *R02F11.3*, and *F56C9.3*) of the six CDFs that do not already have existing null alleles using CRISPR-Cas9 (Au *et al.* 2019; Norris *et al.* 2015). We also obtained a *PDB1.1* deletion mutant, *ok3114*, from the Caenorhabditis Genetics Centre (Barstead *et al.* 2012). Additionally, we generated double and triple mutants of intestinally expressed CDFs *ZK185.5*, *K07G5.5*, and *F41C6.7* through genetic crosses, since paralogous genes can work redundantly to serve the same or similar functions. Considering most SLC-related Mendelian disorders are a consequence of loss-of-function mutations (Rives *et al.* 2017), these mutants would also serve as a useful proxy for the effects of an antagonistic drug under basal conditions.

We examined the CDF null mutants for gross phenotypic defects and were not able to detect any discernable morphological, developmental, or locomotory abnormalities under normal culture conditions. To determine whether CDFs also contribute to progeny survival, as seen in

SLC30A4 mutant mice (Huang and Gitschier 1997), we assessed the overall brood size of all five single CRISPR knock-out mutants and a *ZK185.5* (*gk5056*); *K07G5.5* (*gk5085*) double mutant. Brood sizes amongst CDF mutants were not significantly different from N2 wildtype broods (Figure 3.3) and their progeny were able to develop normally until at least L4 (data not shown). Though these assessments indicate that CDFs in *C. elegans* are not essential for survival under lab culture conditions, elucidating the biological roles of these transporters would positively contribute to our understanding of helminth-biology and metal homeostasis.

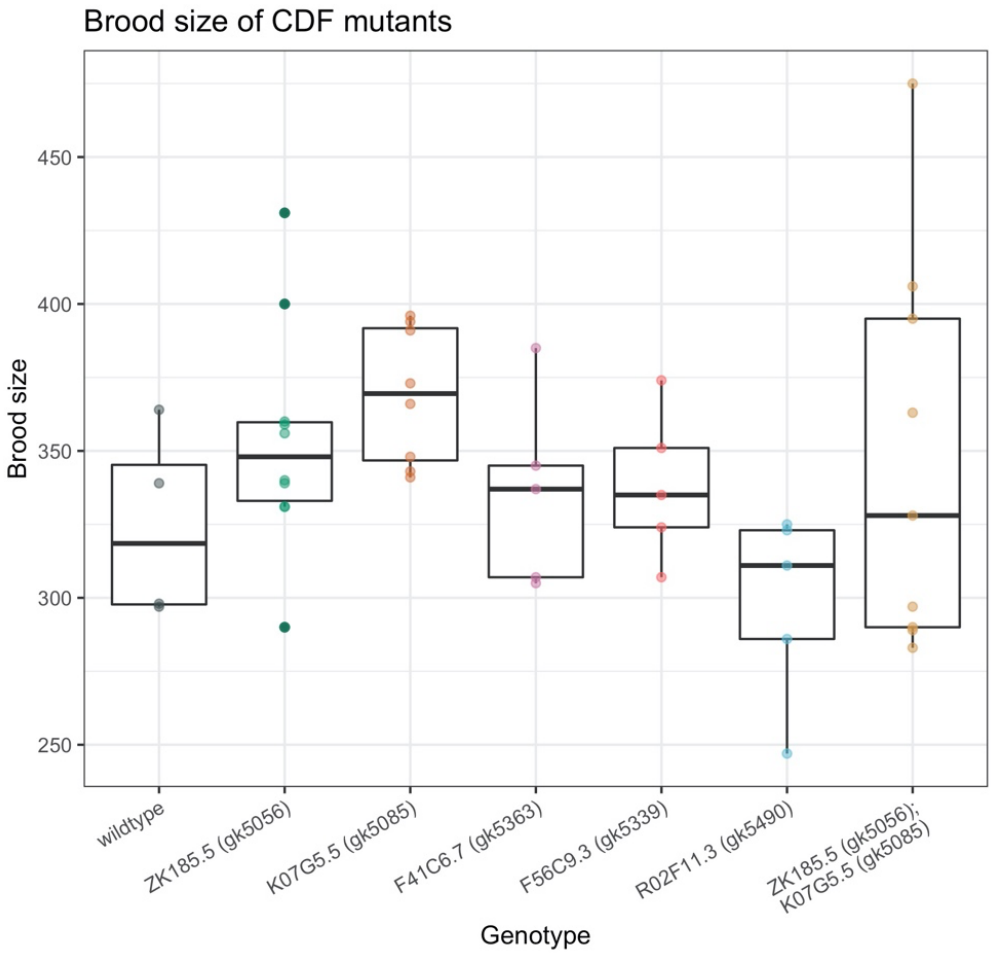

Figure 3.3 Helminth-specific CDF null mutations do not affect overall brood size. Wildtype and CDF null were singled at L4-stage onto 60mm NGM plates seeded with OP50 *E.coli*. F_1 progeny were counted over the course of 5 days. A one-way ANOVA test showed CDF null mutations, even in combination, did not have an effect on brood size, $F(6,39) = 1.764$, $p = 0.132$.

3.3 Manganese tolerance is regulated by novel *C. elegans* cation diffusion facilitators

There are 14 predicted CDFs in *C. elegans*. Several phylogenetic analyses of CDFs indicate that "nematode-specific" efflux transporters found in *C. elegans* are more closely related to manganese transporters found in *Arabidopsis thaliana* (AtMTP11) and *Stylosanthes hamata* (ShMTP1) than other CDFs found in *C. elegans* and humans (Roh *et al.* 2013; Cubillas *et al.* 2013; Montanini *et al.* 2007; unpublished data from Dr. Cubillas, Washington University, St. Louis). Alongside our genes of interest, we also see predicted CDFs of parasitic helminths from both Nematoda (i.e. *Ascaris suum, Loa loa*, and *Brugia malayi*) and Platyhelminthes (i.e. *Schistosoma mansoni* and *Clonorchis sinensis*) cluster within the same phylogenetic branch (unpublished data from Dr. Ciro Cubillas not shown). These phylogenies suggest that our "nematode-specific" CDFs are related to a variety of helminths and may function to mobilize manganese out of the cytosolic space to mediate manganese homeostasis.

To examine the substrate specificity of these putative manganese transporters in *C. elegans*, we collaborated with Dr. Ciro Cubillas from the Kornfeld Lab (Washington University, St. Louis, U.S.A.) to test the metal sensitivity of *PDB1.1* (*ok3114*), and the five CDF null mutants generated using CRISPR-Cas9 under a variety of extreme metal conditions. We began with synchronized L1 larvae of CDF mutants grown on noble agar minimal media (NAMM) plates with high concentrations of supplemental metals (500 μM, 300 μM Cu, and 100 μM Zn) and a control condition with no supplemental metal provided. After three days, the worms were immobilized with tetramisole hydrochloride and mounted on slides, imaged with a ZEISS Axioplan 2 microscope, and measured with ImageJ (Schneider *et al.* 2012). We used relative body length (body length / mean body length at control condition) as a measure of growth to compare the responses of different CDF mutants to N2 across various metal conditions.

Of the six CDFs tested, only *R02F11.3* (*gk5490*) and *PDB1.1* (*ok3114*) showed a significant reduction in body size at 500 µM Mn as compared to wild-type and did not show increased sensitivity to other metal species relative to wild-type response (Figure 3.4A). This suggests that *R02F11.3* and *PDB1.1* both function as manganese-selective efflux transporters that contribute to manganese tolerance or homeostasis. One CDF, *K07G5.5* (*gk5085*), showed increased sensitivity to higher manganese concentrations at 750 µM Mn (Figure 3.5).

Based on single-cell combinatorial indexing sequencing data, *ZK185.5*, *K07G5.5*, *PDB1.1*, and *F41C6.7* are all expressed in the intestine (Hutter and Suh 2016; Cao *et al.* 2017). To see if these proteins act as redundant partners, we generated a double (*ZK185.5; K07G5.5*) and a triple (*ZK185.5*; *K07G5.5*; *F41C6.7*) CDF mutant, and exposed them to 500 µM and 750 µM supplemental Mn conditions. Both double and triple mutants are more sensitive to manganese than their single knock-out counterparts at 750 µM supplemental Mn (Figure 3.6).

A

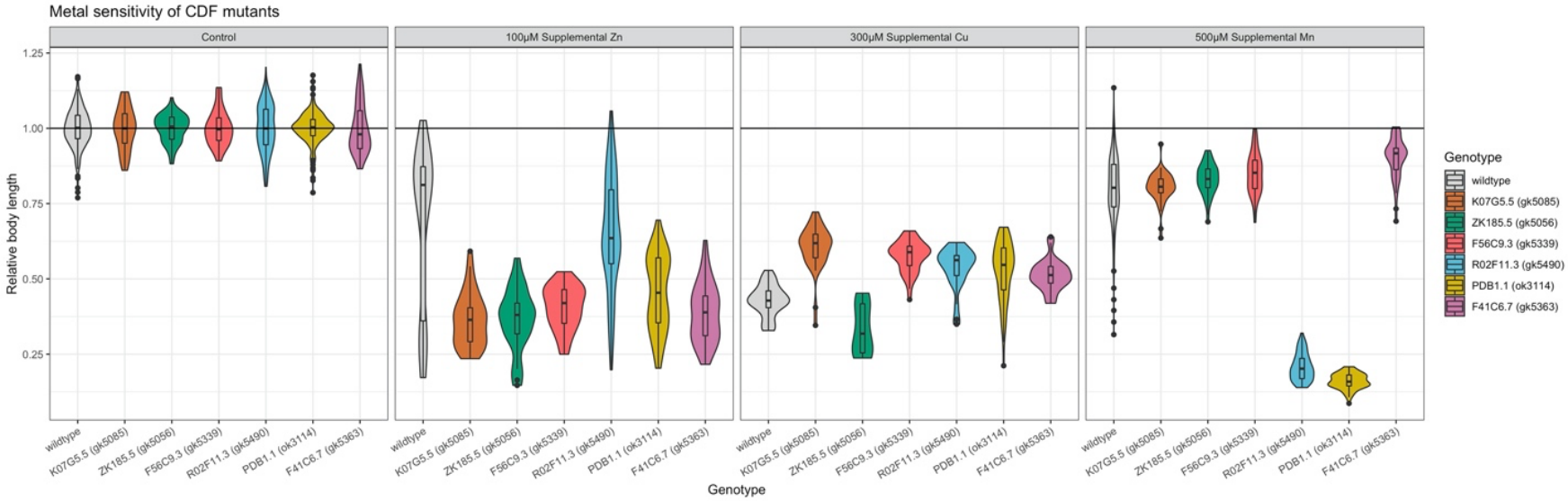

Figure 3.4 *R02F11.3* and *PDB1.1* null mutants are sensitive to high manganese conditions. **(A)** Two CDF mutants, *R02F11.3* (*gk5490*) and *PDB1.1* (*ok3114*), show a significant reduction in body length at 500µM Mn as compared to N2 wildtype at the same condition (Tukey's HSD, $p \leq 0.0001$****).

B

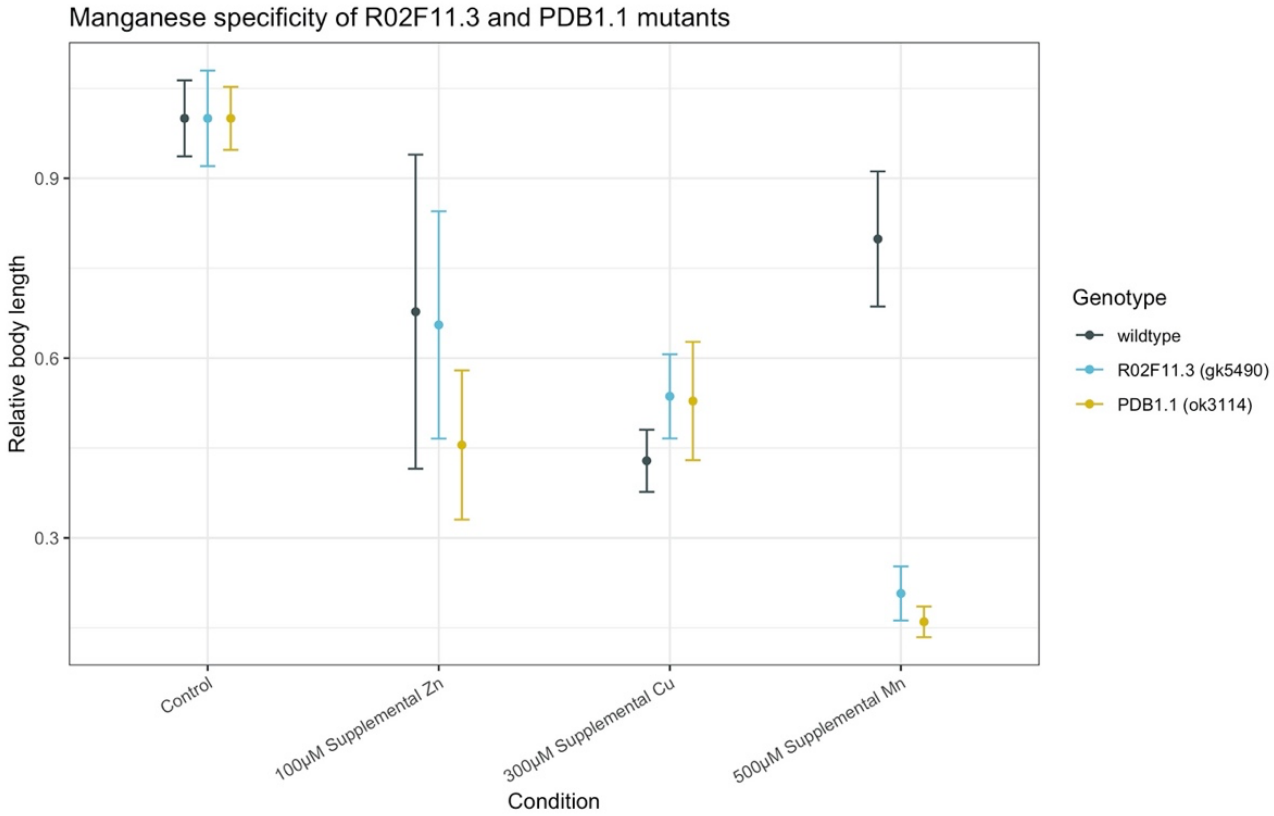

Figure 3.4 *R02F11.3* and *PDB1.1* null mutants are sensitive to high manganese conditions. **(B)** Both *R02F11.3* (*gk5490*) and *PDB1.1* (*ok3114*) responses are specific to the 500µM supplemental Mn condition, and are not observed on 100µM supplemental Zn or 300µM supplemental Cu plates. The points show mean relative body length and the error bars are standard deviations. On average, N2 shows a 13% decrease in body length at 500µM Mn as compared to control condition, while *R02F11.3* (*gk5490*) and *PDB1.1* (*ok3114*) show an 80% and 84% reduction in body length, respectively, at 500µM Mn relative to control condition.

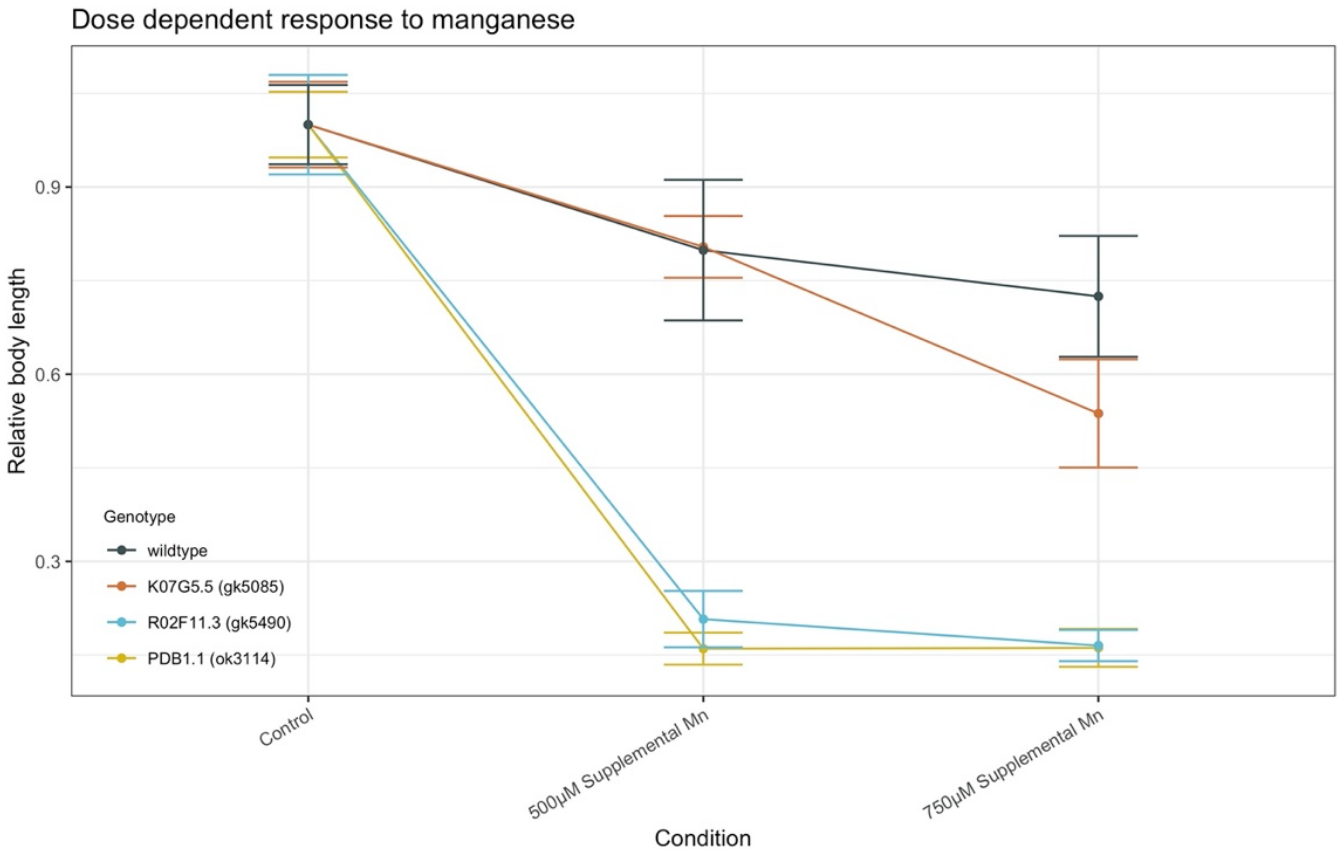

Figure 3.5 *K07G5.5* (*gk5085*) shows increased sensitivity from 500µM Mn supplemental Mn to 750µM supplemental Mn (student's t-test, $p \leq 0.0001$****). The points show mean relative body size and the error bars are standard deviation.

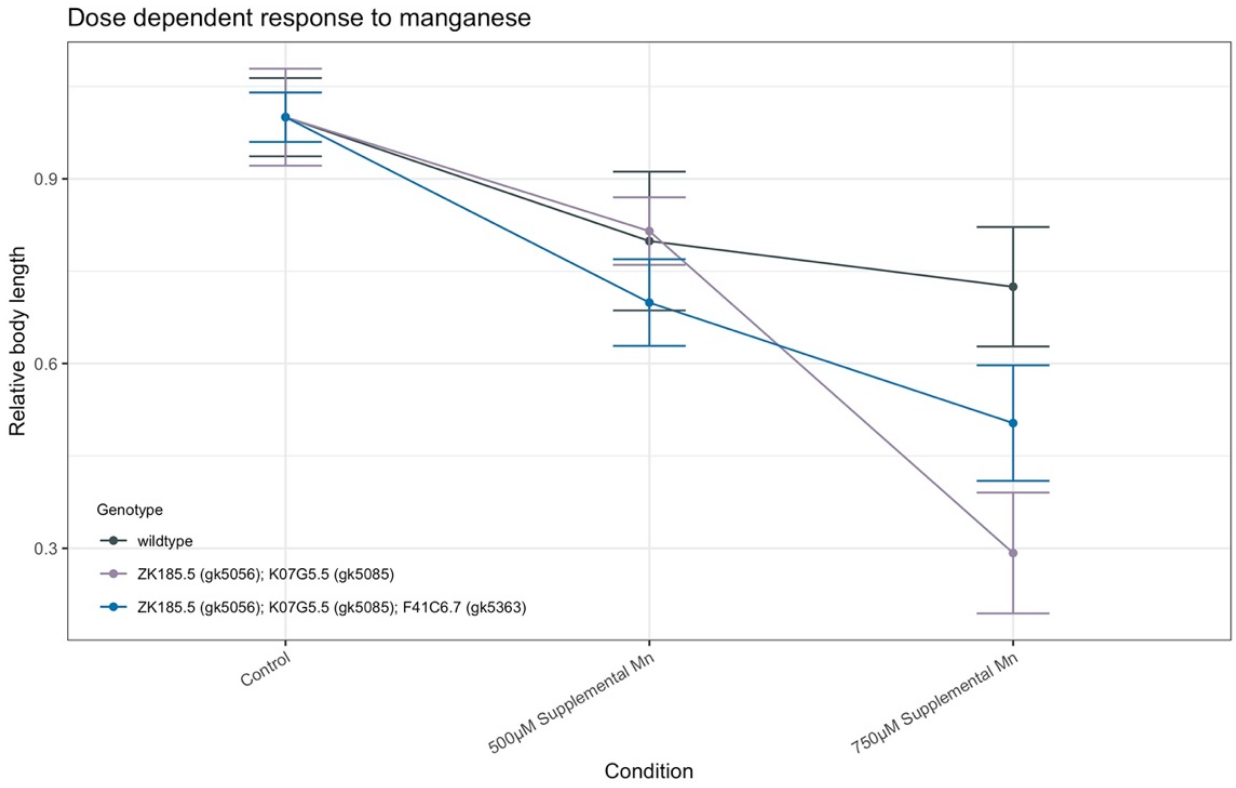

Figure 3.6 Intestinal CDFs work redundantly to maintain manganese homeostasis. The double mutant, *ZK185.5* (*gk5056*); *K07G5.5* (*gk5085*), is more resilient to manganese than the triple mutant, *ZK185.5* (*gk5056*); *K07G5.5* (*gk5085*); *F41C6.7*(*gk5363*) (Tukey's HSD, $p \leq 0.0001$****). This indicates that *K07G5.5* and *ZK185.5* may be working together in parallel pathways to achieve manganese detoxification, while *F41C6.7* may move manganese into areas more sensitive to the toxic effects of high manganese concentrations. The points show mean relative body size and the error bars are standard deviations.

To begin to illuminate the relationship between manganese dysregulation and body length, we carefully observed *PDB1.1* (*ok3114*) and *R02F11.3* (*gk5490*) on NAMM plates with 500 μM supplemental Mn over the course of three days. Even after three days under high manganese condition, the two null mutants remain developmentally stalled at the L1/L2 larval stage (see Appendix C). For both CDF knockouts, many worms looked sickly (i.e. slow-moving and clear with disorganized guts) or were presumed dead (i.e. unresponsive to touch) after 3 days of exposure to high manganese conditions. In contrast, wildtype worms developed relatively normally under the same extreme conditions—though they also seem to be shorter in length that their counterparts grown on regular NGM plates. To see if this effect was reversible, we transferred the stalled worms onto regular NGM plates after two days of exposure at 500 μM Mn and over 70% of worms resumed development after returning to basal conditions for one day (data not shown). However, the longer they are left under high manganese conditions, the longer it takes for them to return to normal development and the more sickly they become as adults—presumably the result of manganese-mediated oxidative stress, mitochondrial disruption (Settivari *et al.* 2009), and/or protein dyshomeostasis (Angeli *et al.* 2014). This demonstrates that manganese negatively affects growth and that the effect is partially reversible.

3.4 Subcellular localization of intestinal manganese cation diffusion facilitators

Although CDFs are expressed across a diverse number of tissue in *C. elegans*, the majority of them are found in the intestine (Table 3.4). Even within the same cell, we see different CDFs (and even different isoforms of the same CDF) localize to discrete membranous regions (Table 3.4). For example, zinc-transporting CDFs are known to localize at the apical (Roh *et al.* 2013) and basolateral (Bruinsma *et al.* 2002) surface of intestinal cells, as well the plasma membrane of intestinal gut granules (Davis *et al.* 2009). By illuminating where these

efflux transporter are located, we can start to construct a model for how metals are eliminated, sequestered, and mobilized for detoxification, storage, and distribution.

Gene name	CDS name	Metal substrate	Tissue expression (most highly expressed, top 3)†	Subcellular Localization	Reference
cdf-1	C15B12.7	Zn	Intestine, interneurons, pharyngeal muscle	Basolateral membrane of intestinal cells and in membrane of vulval cells	Bruinsma *et al.* (2002)
cdf-2	T18D3.3	Zn	Intestine, body wall muscle*, seam cells*	Plasma membrane of gut granules	Davis *et al.* (2009)
ttm-1a	Y39E4A.2a	Zn	Intestine, canal associated neurons, socket cells	Localizes as puncta in hypodermal and intestinal cells (close to apical surface), but distinct from gut granules and mitochondria	Roh *et al.* (2013)
ttm-1b	Y39E4A.2b	Zn	Intestine, canal associated neurons, socket cells	Apical membrane of intestinal cells	Roh *et al.* (2013)
sur-7	F01G12.2	Zn	Canal associated neurons, intestine, somatic gonad precursors	Endoplasmic reticulum of most tissues (except intestinal cells)	Yoder *et al.* (2004)
TOC-1	ZC395.3	Unknown	Coelomocytes, distal tip cells, pharyngeal gland	Unknown	
F19C6.5	F19C6.5	Unknown	Socket cells*, intestine, germline, body wall muscle	Unknown	
slc-30A9	Y71H2AM.9	Unknown	Intestine, germline, somatic gonad precursors	Unknown	
slc-30A5	Y105E8A.3	Unknown	Intestine, distal tip cells, rectum	Endoplasmic reticulum	Meissner *et al.* (2011) — subcellular localization only
PDB1.1	PDB1.1	Mn	Intestine, Am/PH sheath cells*, pharyngeal epithelia*	Plasma membrane of compartments close to apical surface of intestinal cells and co-localizes with gut granules	This thesis work in collaboration with Dr. Ciro Cubillas at the Kornfeld Lab
F41C6.7	F41C6.7	Mn?	Intestine, non-seam hypodermis*, seam cells	Both apical and basal lateral membrane of intestinal cells	This thesis work in collaboration with Dr. Ciro Cubillas at the Kornfeld Lab

Gene name	CDS name	Metal substrate	Tissue expression (most highly expressed, top 3)†	Subcellular Localization	Reference
K07G5.5	K07G5.5	Mn?	Intestine, pharyngeal muscle, seam cells	Unknown	This thesis work in collaboration with Dr. Ciro Cubillas at the Kornfeld Lab
ZK185.5	ZK185.5	Mn?	Intestine, intestinal/rectal muscle*, coelomocytes*	Unknown	This thesis work in collaboration with Dr. Ciro Cubillas at the Kornfeld Lab
R02F11.3	R02F11.3	Mn	Excretory cells, sex myoblasts, GABAergic neurons	Unknown	This thesis work in collaboration with Dr. Ciro Cubillas at the Kornfeld Lab
F56C9.3	F56C9.3	Unknown	Germline, distal tip cells, Am/PH shealth cells	Unknown	

† Based on single-cell RNA expression of L2-staged N2 worms from Cao *et al.* (2017), retrieved from gExplore (genome.sfu.ca/gexplore; Hutter and Suh 2016)

* Indicates tissue expression level based on only a small number of reads (Hutter and Suh 2016)

Table 3.2 Localization of cation diffusion facilitators. CDFs in *C. elegans* are predominantly expressed within the intestine and localize to a variety of membranes.

To determine the precise localization of the CDFs in this study, we chose to tag the genes with either GFP or mCherry using CRISPR-Cas9 methodology developed by Norris *et al.* (2015). By tagging the endogenous proteins, we were able to overcome common confounding factors sometimes seen in fluorescently tagged proteins overexpressed from extrachromosomal arrays; these include mosaic expression, silencing, aberrant localization, and even toxicity due to over-expression (Praitis *et al.* 2001). For two members of the manganese CDF family, *F41C6.7* and *ZK185.5*, we tagged the genes at the C-terminus with GFP using CRISPR-Cas9. We also tagged *PDB1.1* at the C-terminus with mCherry which should, in theory, allowed us to visualize all three isoforms because they share the same transcriptional termination site.

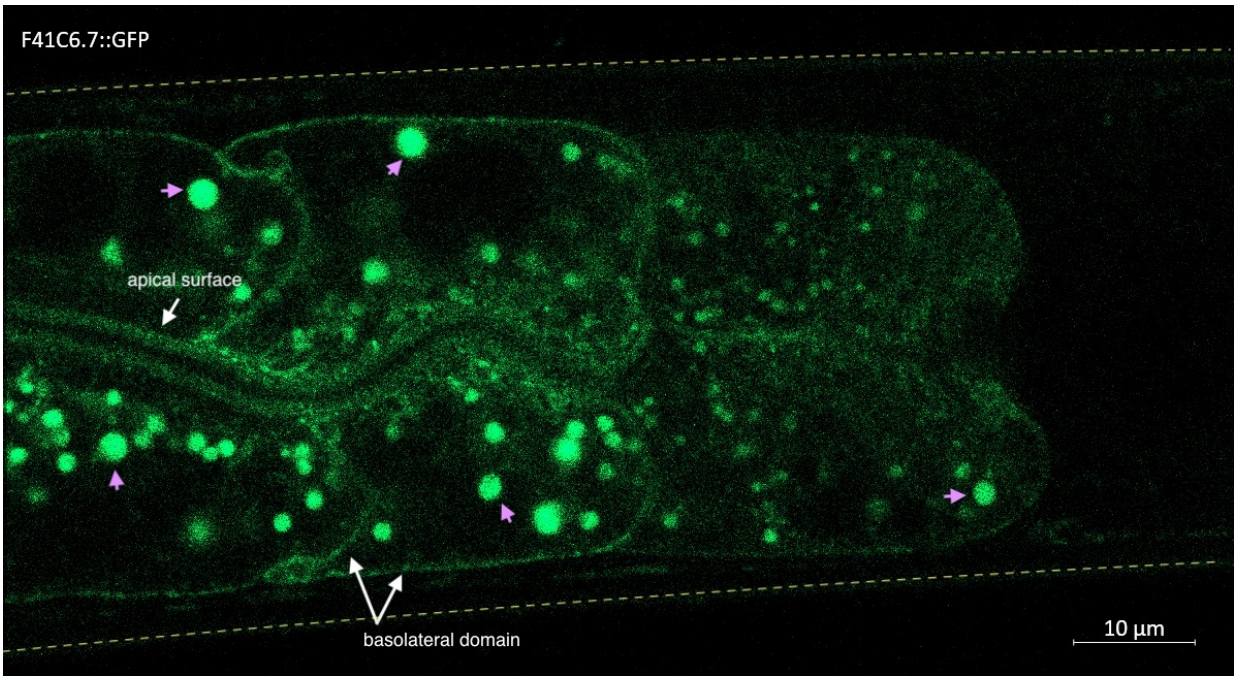

Figure 3.5 Localization of F41C6.7::GFP. F41C6.7::GFP localizes to the membrane of intestinal cells and outlines individual intestinal cells (int1 to int3). Worms imaged were staged as mid-L4 (L4.3-L4.6) by vulval development (Mok *et al.* 2015). The anterior of the worm is to the right. The round vesicles are autofluorescent gut granules (purple arrows). Body wall is indicated by the dotted yellow line.

F41C6.7::GFP appears to localize at the intestinal cell membrane, at the apical surface as well as the basolateral domain, and may be congregating at the brush border as suggested by the thick GFP outline of the lumen (Figure 3.7).

PDB1.1*::*mCherry appear to localize in discrete puncta near the intestinal lumen that are distinct from autofluorescent gut granules (Figure 3.8). However, since autofluorescent gut granules can also be seen under the RFP filter in a PD1074 wildtype background, we cannot tell whether PDB1.1::mCherry also colocalizes with gut granules as well. Since PDB1.1 has multiple isoforms, it is conceivable that they localize to different parts of the cell, similar to TTM-1 (Roh *et al.* 2013). It has also been shown that Zn-CDFs use gut granules as storage sites (Davis *et al.* 2009), and Mn-CDFs may be using a similar strategy.

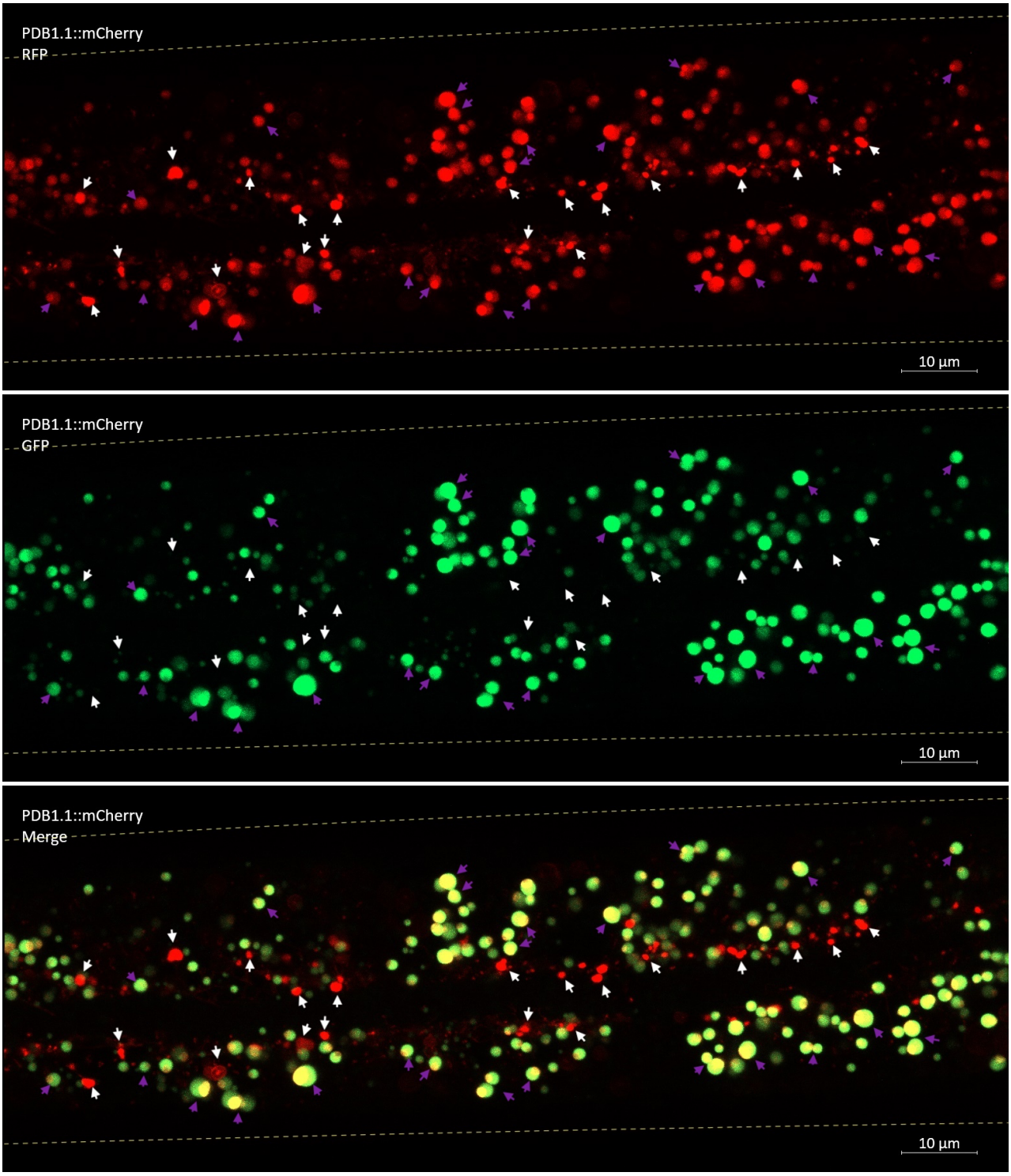

Figure 3.6 PDB1.1::mCherry appears in distinct puncta near intestinal lumen. Top: PDB1.1::mCherry and autofluorescent gut granule expression in red . Middle: autofluorescent gut granules in green. Gut granules (highlighted by purple arrowheads) appear under both GFP and RFP filters. Bottom:

PDB1.1::mCherry also appears in distinct puncta that do not overlap with gut granules and congregate near the apical surface of intestinal cells (examples indicated by white arrowheads).

We were not able to visualize ZK185.5::GFP under the confocal microscope. However, we were able to construct a P*ZK185.5*::GFPnovo2 transcriptional reporter and was able to see GFP expression from an extrachromosomal array. Expression of *ZK185.5* was present all along the intestine, but was most strongly expressed near the utmost anterior and posterior regions of the intestinal tract (Figure 3.9).

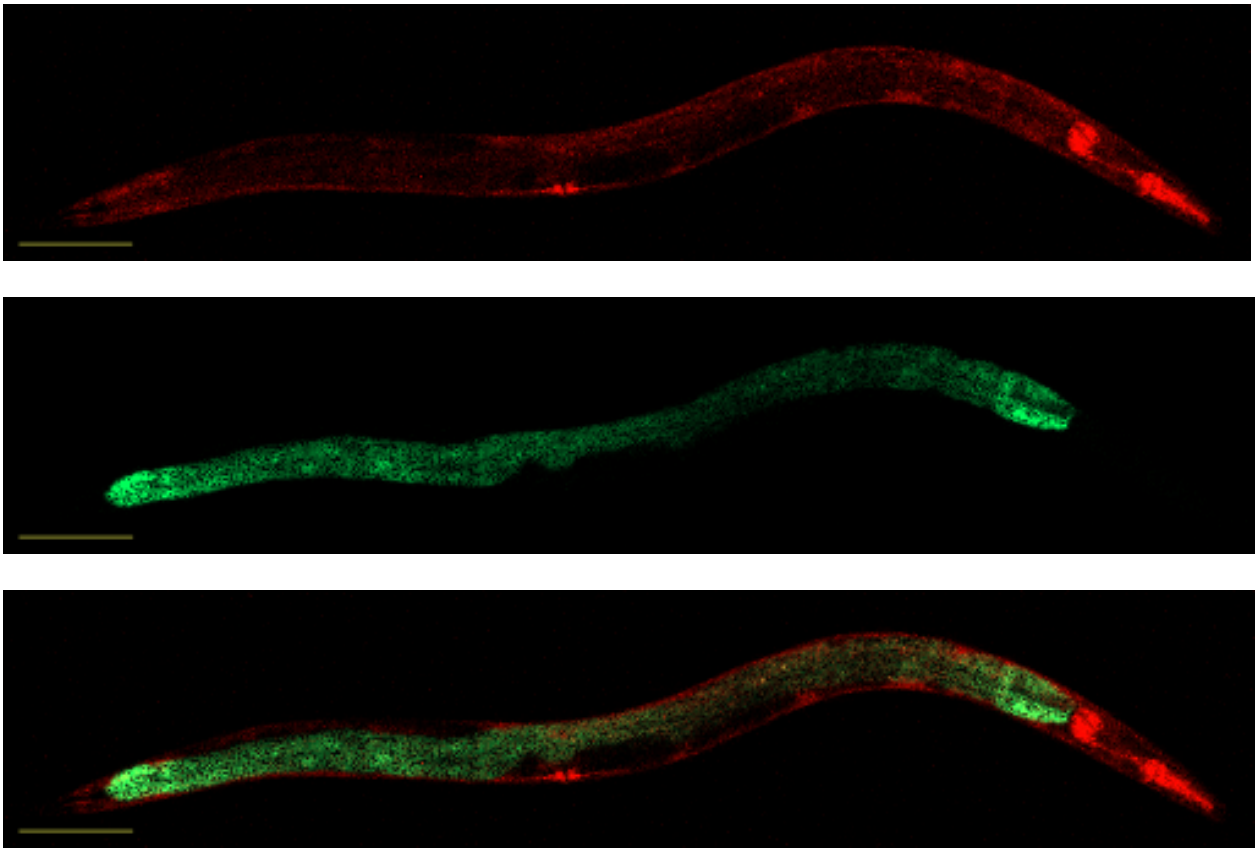

Figure 3.7 *ZK185.5* is expressed in the intestine. (top) Body-wall and pharyngeal muscle mCherry expression from P*myo-3*::mCherry and *Pmyo-2*::mCherry extrachromosomal arrays, respectively. (middle) Transcriptional reporter, P*ZK185.5*::GFP, shows *ZK185.5* expression in green.

We also attempted to express R02F11.3::eGFP from a fosmid clone (CBGtg9050H0397D) provided by the SourceBioscience's *C. elegans* TransgenOme Resource (Sarov *et al.* 2012, www.sourcebioscience.com), but we were not able see any fluorescence from multiple injections of the construct.

3.5 *R02F11.3* null mutation does not damage dopaminergic neurons under normal growth conditions

It has been well-documented that chronic exposure to environmental manganese can be neurotoxic and may lead to manganism, a condition that presents similarly to Parkinson's disease (Avila *et al.* 2013). Parkinson's disease is a disorder caused by degenerating dopaminergic neurons (Angeli *et al.* 2014), and previous studies have shown that dopaminergic neurons are vulnerable to manganese-induced neurotoxicity in humans (Lin *et al.* 2020; Stanwood et al 2009). The prevailing theory is that manganese mediates dopamine oxidation which leads to the generation of noxious reactive oxygen species that damage neurons (Harischandra *et al.* 2019; Benedetto *et al.* 2010). Indeed when *C. elegans* is challenged with acute exposure to manganese, we also see damage of dopaminergic neurons as well (Benedetto *et al.* 2010). Moreover, mutations in human manganese efflux transporter SLC30A10 causes familial manganism and accumulation of manganese even without the introduction of excess manganese through either diet or the environment (Tuschl *et al.* 2008; Tuschl *et al.* 2012; Peres *et al.* 2016). Since *R02F11.3* is expressed in ciliated sensory neurons (Hutter and Soh 2016; Cao et al 2017), we decided to look for possible defects in the CEP and ADE ciliated mechanosensory dopaminergic neurons under basal conditions of *R02F11.3*(*gk5490*) mutants under normal conditions using a transcriptional reporter of dopamine transporter *dat-1* (Pdat-1::GFP) that allowed us to visualize all eight dopaminergic neurons in the worm: 4 CEP, 2 ADE, and 2 PDE neurons.

Benedetto *et al.* (2010) reported some evidence of neuronal degeneration judged by breaks in GFP signal in the CEP and ADE neurons after acute exposure to manganese for 30 min starting at 500µM Mn. If *R02F11.3* were unable to export manganese out of CEP and ADE neurons, we postulate that they may also suffer manganese-induced dopaminergic damage. We crossed Pdat-1::GFP worms with *R02F11.3* (*gk5490*) and looked for evidence of neuronal damage in CEP and ADE head neurons in homozygous *gk5490* animals at basal conditions. We were unable to see any signs of dopaminergic neuron damage (Figure 3.5), however it still remains to be seen whether *R02F11.3* (*gk5490*) is more sensitized to manganese-induced injury than wildtype.

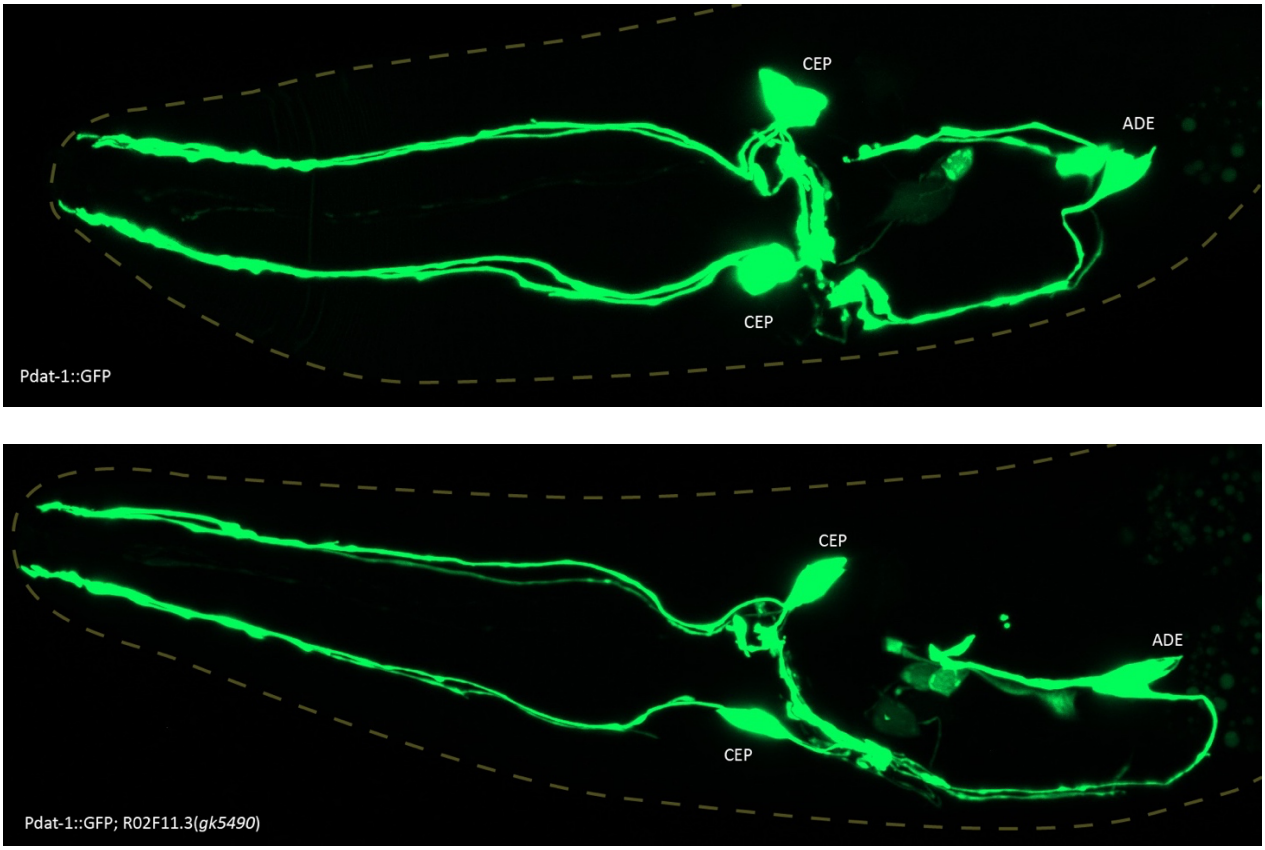

Figure 3.8 *R02F11.3* (*gk5490*) does not exhibit neuronal damage in dopaminergic head neurons, CEP and ADE, under basal condition. The dotted line shows the outline of the worm.

Chapter 4: Discussion

4.1 A world of "nematode-specific" and druggable orthologs

In this study, we identified 374 "nematode-specific" orthologs present across 11 free-living and parasitic nematode species. These 374 orthologs are absent from 5 outgroup species and humans using WormBase Parasite (WBP, Version 13.0) BioMart. However, this pipeline does present some limitations. As multiple genome assemblies exist for certain helminth species, the number of orthologs may change depending on the genome assembly selected for the analysis. Furthermore, genome annotations are reliant on gene prediction programs. Any list of putatively druggable proteins will evolve to reflect incremental improvements on the WBP database and gene prediction algorithm. In addition, WBP BioMart relies on Ensembl Compara as their sole orthology prediction algorithm. Previous studies have shown that different prediction methods will produce different results (Pereira *et al.* 2014; Chen *et al.* 2007). Since our initial list of orthologs are produced using Ensembl Compara alone, we may have overlooked orthologs that would have otherwise been predicted by another algorithm, overestimate the number of "nematode-specific" genes by failing to identify orthologs present in our outgroup, or a mix of both. Despite the shifting sands of the genome revolution, we still maintain that orthology predictions are still quite useful at predicting well-conserved (and therefore presumably important) genes—especially when the candidates in question are found across eleven species. For the purpose of this study we considered these genes "nematode-specific", but some of them share orthologs with species not included in the initial analysis. We will explore in the next section how some orthologs found in nematodes, specifically CDFs, are also found in plants, fungi, protists, and platyhelminths as well.

In a first step to determine whether these "nematode-specific" orthologs are suitable drug targets, we identified proteins that are considered "druggable". We filtered the 373 orthologs for ones that were identified as either part of the kinase, GPCR, ion channel, or SLC protein family and found 33 putative druggable proteins. This list can be expanded by including additional druggable families or proteins involved in critical metabolic pathways. Proteases, for example, are an established family of drug targets (Hopkins and Groom 2002) that can be potentially perturbed by anthelmintic drugs. These enzymes are necessary for parasite invasion of host tissue (Grote *et al.* 2018), evasion of the host immune system through immunosuppression (Yang *et al.* 2015), digestion (Tandon *et al.* 2017), and more. Indeed, studies have identified a cysteine protease inhibitor (K11777) able to treat hookworm and other helminth infections in animal models, though the mechanism of action is not helminth-specific (Vermeire *et al.* 2012; Abdulla *et al.* 2007). Previous studies have also demonstrated functional conservation in *C. elegans* through gene rescue with orthologous parasitic nematode proteases involved in moulting (Page *et al.* 2014) and embryogenesis (Britton and Murray 2002). However, the number of proteases that are predicted to both be essential and divergent enough from host proteins to be drug targets are few (Tandon *et al.* 2017). Identifying metabolic chokepoints can also help reveal additional novel targets (Taylor *et al.* 2013). Anaerobic metabolism, specifically the production of electron carrier rhodoquinone, is required for parasitic helminths to survive the anoxic environment of host tissue and for *C. elegans* to adapt to hypoxic conditions; this may prove to be a promising new avenue for anthelmintic drug discovery (Del Borello *et al.* 2019; Buceta *et al.* 2019). There are a number of other protein families that can also be explored to identify targets that span the diversity of the helminths, but we chose to limit our focus to one specific group for the purpose of this small exploratory study.

From the list of 33 putative druggable proteins, we focused on two paralogs predicted to be CDFs involved in metal homeostasis—a process critical for the health and survival of all organisms. We expanded our investigation to include their paralogs (in case of functional redundancy) and resolved to elucidate the biological roles of all six CDFs. Alignment of the "nematode-specific" CDFs show less than 22% sequence identity with the closest human ortholog: SLC30A9. Although the sequence identity is quite low, there is a small possibility for compounds to inhibit both nematode and human CDFs given enough structural similarities in binding pockets. To examine this more closely, we proposed using BIOVIA Discovery Studio 4.5 (Dassault Systèmes, Courbevoie, France) software for homology modeling to predict the structure of *C. elegans* CDFs based on the existing crystallized structure of an *E. coli* zinc transporter, YiiP (Lu and Fu, 2007; Lu *et al.* 2009). Unfortunately, the COVID-19 pandemic has curbed our plans to pursue this line of inquiry before the completion of this Master's project.

4.2 Cation diffusion facilitators across domains

Cation diffusion facilitators are found across every kingdom. These metal-specific efflux transporters are responsible for moving one or more metal substrates across membranes. In a recent and expanded phylogenetic analysis using over 10,700 predicted CDF proteins from the Protein Data Bank (PDB), Dr. Ciro Cubillas showed that the six "nematode-specific" CDFs are most closely related to manganese-transporting CDFs found in *A. thaliana* (AtMTP11) and *S. hamata* (ShMTP8, previously named ShMTP1) (unpublished data). Other studies that have attempted to predict the substrate specificity of CDFs through phylogenetic means have also concluded the same relationship (Cubillas *et al.* 2013; Montanini *et al.* 2007). This phylogenetic branch is separate and distinct from the branch that includes previously characterized zinc-transporting CDFs in *C. elegans*. Other members represented within this phylogenetic branch

include mostly species from Dikarya (i.e. fungi), members of Bikonta (e.g. plant, algae, and diatoms), and primitive metazoans (e.g. cnidaria, sea sponges, nematodes, and platyhelminths), but not prokaryotes or more complex eukaryotes (such as chordates)—perhaps suggesting these transporters could have emerged or appeared as a unique group of transporters within Eukaryota.

It is conceivable that horizontal gene transfer (HGT) could have occurred between kingdoms as putative Mn-CDF orthologs are present in distant plant, fungi, and nematode species (and in no other phylums of Ecdysozoa like Arthopoda, unpublished work by Dr. Cubillas). There is a body of genomic and functional evidence that shows HGT is not uncommon within the rhizosphere between bacteria (or other vectors) and nematodes (Danchin *et al.* 2010; Danchin 2011; Danchin *et al.* 2016; Haegeman *et al.* 2011; Mayer *et al.* 2011; Mitreva *et al.* 2009; Nagayasu *et al.* 2013; Wu *et al.* 2013). An interesting example of HGT in nematodes is the acquisition of ferrochelatase from alpha-proteobacteria (Nagayasu *et al.* 2013; Wu *et al.* 2013). Ferrochelatase (FeCH) is the final enzyme required for the biosynthesis of heme, a molecule required for nearly every living organism to support myriad essential functions including oxygen and electron transport (Ferreria *et al.* 1995). Although nematodes do not synthesize their own heme (Hieb *et al.* 1970; Rao *et al.* 2005), genomic and phylogenetic analyses indicates that nematode FeCH are nevertheless present and (for clades III and IV) likely adopted from bacterium (Nagayasu *et al.* 2013; Wu *et al.* 2013). Wu *et al.* (2013) identified that the transferred FeCH had evolved to include hallmarks of eukaryotic genes such as introns and an N-terminal localization signal, and demonstrated that the enzyme is both functional and essential for *B. malayi* embryogenesis. In another independent study, Nagayasu *et al.* (2013) also demonstrated that *Strongyloides venezuelensis* (*Sv*) FeCH is functional through rescue of an *E. coli* hemH null-mutant with *Sv*FeCH. Although Mn-CDFs do not seem to be orthologous with bacteria, it is still

possible for HGT to occur between eukaryotic organisms. The first report of gene transfer between plants and animals (i.e. the whitefly, *Bemisia tabaci*) was published in 2021 by Xia *et al.* They demonstrated that *BtPMaT1*, an enzyme found in *B. tabaci*, is most closely related to plant phenolic glucoside malonyltransferases and is functionally conserved with the plant homologs (Xia *et al.* 2021). When *BtPMaT1* is knocked down by RNAi in whiteflies, the animals show increased toxicity to phenolic glucosides produced by plants (Xia *et al.* 2021). If HGT from plants did indeed seed evolutionarily distinct CDFs in nematodes, then the event must have occurred early enough to be spread across multiple clades of the Nematoda phylum. The most recently constructed cross-domain phylogeny of CDFs does indicate that the origins of manganese CDFs is polyphyletic (Cubillas *et al.* 2013; Kolaj-Robin *et al.* 2015). For future directions, we will be investigating possible phylogenetic incongruencies (Ravenhall *et al.* 2015) between the rate of evolution of CDFs and the species from which the CDF sequences were obtained.

Since its first description in the 1990's (Nies and Silver 1995), CDF phylogenetic ancestry has been explored both across domains (Paulsen and Saier 1997; Cubillas *et al.* 2013; Montanini *et al.* 2007) and within several taxonomic groups (Gustin *et al.* 2011; Ibuot *et al.* 2020). Elucidating an accurate phylogeny remains an important line of inquiry for the metallomics field as it helps predict metal specificity of the many uncharacterized CDFs that remain. Studies of CDFs continue to overturn our previously conceived notions of what they accomplish. Often thought to exclusively move cations out of the cytoplasmic space, detailed studies of individual CDFs have surprised us by yielding other roles: *S. cerevisiae* MMT1 and MMT2 increase cytoplasmic iron by drawing from stores in the mitochondria (Li and Kaplan 1997) while metal transporters in *A. thaliana* (Bloß *et al.* 2002) and a *Synechocystis sp.* (Jiang *et*

al. 2012) both facilitate uptake of metals into the cell. With every functional assessment of previously uncharacterized CDFs, we illuminate a bit more of the picture for this ubiquitous transporter.

4.3 Window into CDF-mediated manganese homeostasis in *C. elegans*

Heavy metals are required for many essential processes of life. The presence of CDFs in every living domain speaks to their importance in the regulation of trace metals through excretion, distribution, storage, and sometimes uptake of cations. Although much attention has been paid to zinc-transporting CDFs (Palmiter and Huang 2004; Huang and Tepaamorndech 2013), very little is known about manganese-selective CDFs in humans or animal models (Chen *et al.* 2015a; Avila *et al.* 2013). In collaboration with Dr. Cubillas and the Kornfeld Lab, we have identified two novel putative manganese-selective CDFs that play a critical role in manganese detoxification: PDB1.1 and R02F11.3.

While wildtype worms develop normally under high manganese conditions (500µM Mn), null mutants PDB1.1 (*ok3114*) and R02F11.3 (*gk5490*) remain developmentally stunted at early larval stages (as measured by body size) with some failing to survive the toxicity. K07G5.5 (*gk5085*) also showed a reduced body size relative to wildtype, but only when exposed to 750µM Mn. In fact, all strains tested in this study (including wildtype) showed at least a modest reduction in body size under high manganese conditions. Previous studies by Xiao *et al.* (2009) and Angeli *et al.* (2014) also reported reduced body size in wildtype worms when challenged with high concentrations of manganese. The remaining three knock-out mutants: F41C6.7 (*gk5363*), ZK185.5 (*gk5056*), and F56C9.3 (*gk5339*), did not show a response significantly different from that of wildtype, even at 750µM Mn. Remarkably, both PDB1.1 (*ok3114*) and R02F11.3 (*gk5490*) were able to resume normal development when returned to regular growth

conditions on NGM plates (this was not investigated in the K07G5.5 null mutant). Although this was not explicitly assayed in this project, we suspect the reversible effect of high manganese toxicity on *C. elegans* Mn-CDF mutants may also be generalized for other transition metals since wildtype worms also exhibit a reduced body size when exposed to high concentrations of zinc and copper. In addition, we observed both CDF mutants grew increasingly sick as they spent more time under high manganese conditions and many ultimately succumb to the toxicity over time. This observation is consistent with previous studies which demonstrated that manganese induces oxidative stress, mitochondrial dysfunction (Settivari *et al.* 2009), and disrupts protein homeostasis (Angeli *et al.* 2014), ultimately leading to unhealthy animals with shortened lifespans. Further study will need to be done to elucidate the underlying mechanisms behind the manganese-induced developmental arrest of PDB1.1 (*ok3114*) and R02F11.3 (*gk5490*), an endeavor that may reveal the roots of a more generalized response to heavy metal toxicity.

The intestine is a major organ of metal detoxification in *C. elegans* and several previously characterized CDFs are known to localize to the intestine (Davis *et al.* 2019; Roh *et al.* 2012; Roh *et al.* 2013). Single-cell transcriptional profiling indicates four of the six "nematode-specific" CDFs are also expressed in the intestine: PDB1.1, K07G5.5, ZK185.5, and F41C6.7 (Cao *et al.* 2017; Hutter and Suh 2016) and may be working redundantly to achieve manganese homeostasis. To discern whether F41C6.7 and ZK185.5 also contributes to manganese detoxification, we tested double and triple mutants of F41C6.7, ZK185.5, and K07G5.5. Surprisingly, our results showed that the triple-mutant is more resilient to high manganese concentrations than the double ZK185.5 and K07G5.5 mutant. One possible interpretation is that K07G5.5 and ZK185.5 serve similar roles in parallel manganese detoxification pathways while F41C6.7 distributes the metal to surrounding tissue (Figure 4.2).

Without K07G5.5 and ZK185.5's ability to discharge or sequester manganese, a high intracellular concentration of manganese may drive F41C6.7 to export the metal to other tissues and exacerbate the damage. This theory is partially supported by confocal images of F41C6.7::GFP which shows the protein localizing at the basolateral intestinal cell membrane (although it's also expressed in the apical membrane as well).

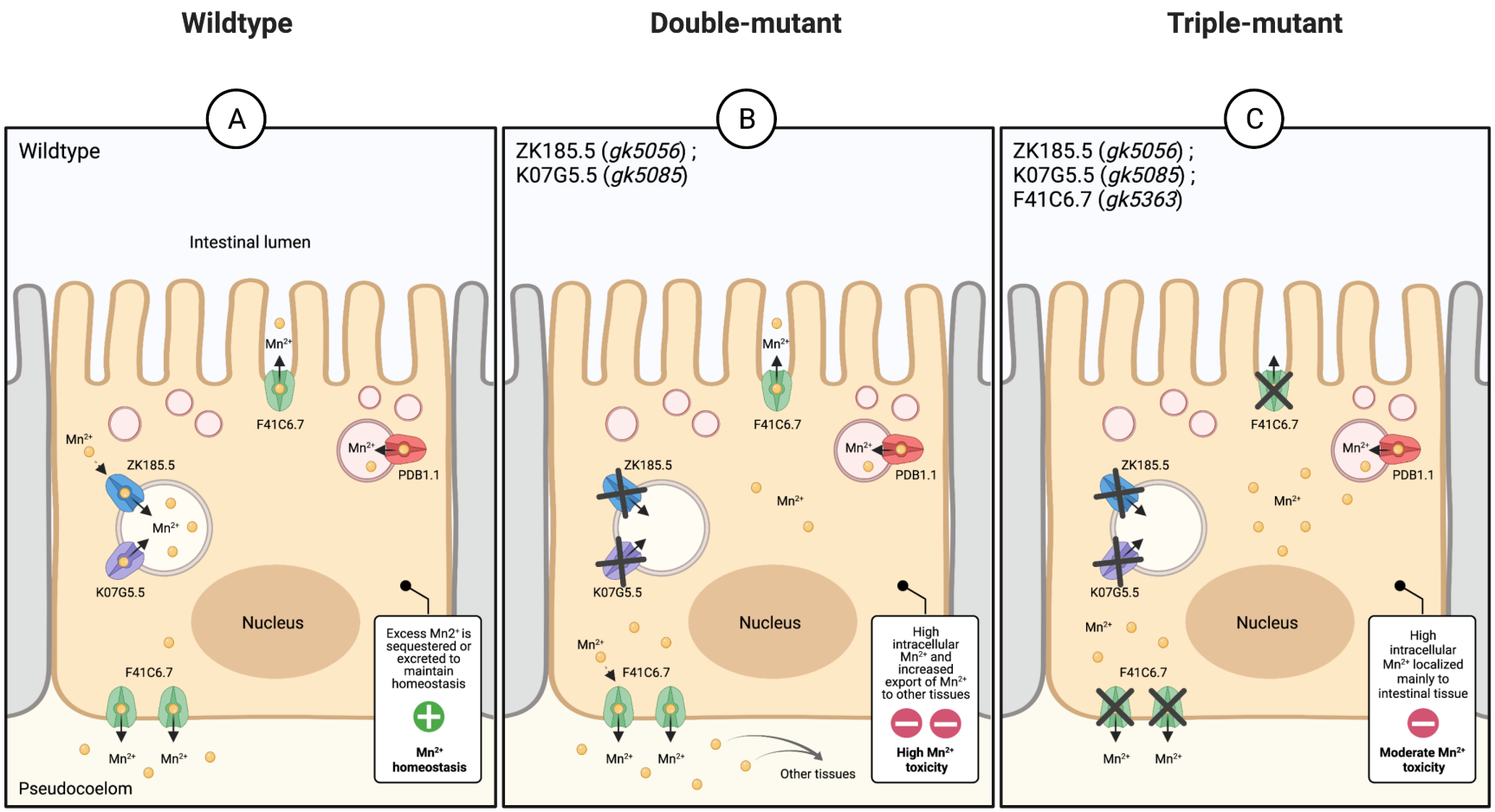

Figure 4.1 Model of Mn-CDF coordination in Mn homeostasis. A model of how movement of manganese may be coordinated by the four intestinal manganese cation diffusion facilitators to maintain homeostasis in *C. elegans*. PDB1.1 transports Mn^{2+} into an unidentified structure that congregates close to the apical surface of the intestinal epithelium and is a major contributor to manganese detoxification. ZK185.5 and K07G5.5 may be working redundantly to sequester manganese in the cell (localization and mechanism unknown) while F41C6.7 distributes to surrounding tissue. Figure created with BioRender.com.

Confocal images of fluorescently-tagged PDB1.1 also shows expression in intestinal cells, validating Cao et. al's (2017) single-cell transcriptomic data set. PDB1.1::mCherry appears in vesicles that congregate close to the intestinal lumen and does not seem to co-localize with gut granules (although this is difficult to tell as the autofluorescence of gut granules can also be seen in wildtype worms imaged under an mCherry filter as well). In a study conducted by the Kornfeld lab, they demonstrated that intestinal CDF-2 sequesters or stores zinc in gut granules to detoxify the worm under high zinc conditions; these gut granules adopt a bilobed (two-lobed) conformation to accommodate zinc in the new lobe (Roh *et al.* 2012). This suggests that PDB1.1 may also play a role in sequestering excess manganese by shuttling it into vesicles. Future experiments should address whether gut granule deficient mutants are similarly sensitized to manganese toxicity to confirm or rule out their contribution to manganese homeostasis and where each of the three transcripts of *PDB1.1* are localized.

A transcriptional reporter of *ZK185.5* also indicates that the gene is expressed in the intestine and is more pronounced in the region just behind the pharynx and by the tail. This observation is consistent with transcriptomics data from Cao *et al.*'s (2017) study. Although we were not able to visualize endogenously tagged ZK185.5 before the completion of this thesis, future attempts to identify where the protein localizes may include antibody staining to GFP, which would greatly enhance the signal.

While *R02F11.3* is not expressed in the intestine, it is evidently still important for manganese detoxification. Expression data shows that it is expressed highly in the excretory system (Cao *et al.* 2017; Hutter and Suh 2016). The excretory system consists of only three tubular cells and is involved in osmoregulation and secretion (Sundaram and Buechner 2016). This may also be a major route of manganese excretion under high manganese conditions.

Our experimental results conclude that both PDB1.1 and R02F11.3 are manganese efflux transporters critical for maintaining manganese homeostasis, likely through sequestration of the cation in intestinal vesicles or expulsion through the excretory system, respectively. In addition, we infer that K07G5.5 and ZK185.5 work redundantly to detoxify *C. elegans* under high manganese conditions. We also postulate that F41C6.7 distributes manganese from intestinal cells to other tissues. Though all five CDFs are implicated in the movement of manganese, it is unclear whether they are all manganese-selective transporters. Since wildtype exhibits a wide range of body sizes in response to high zinc conditions thus overlapping with mutant CDF responses, we cannot rule out the possibility that the CDFs in this study also move zinc. We have implicated five of the six "helminth-specific" CDFs in manganese homeostasis and have observed that manganese has a negative, but reversible, effect on growth and development. To our knowledge, this is the first body of work that has identified and delineated a role for CDFs in manganese homeostasis within *C. elegans*, serving as a springboard for further studies in manganese biology within the worm.

4.4 PDB1.1 may modulate innate immune response

Pathogenic bacteria require transition metals for survival, growth, and virulence. To combat these pathogens, host organisms starve invaders of vital nutrients by employing mechanisms to redistribute, store, or sequester metals—a strategy called nutritional immunity (Brophy and Nolan 2015; Juttukonda and Skaar 2017). In an environment where host and pathogen compete for zinc, iron, and manganese, both have evolved proteins to traffic metals for their own advantage. One well-studied and functionally conserved family of metal transporters involved in innate immunity are the Nramps (natural resistance-associated macrophage proteins). There are three *C. elegans* Nramp homologs: *smf-1*, *smf-2*, and *smf-3*, which are involved in the

uptake of iron and manganese at the intestinal and pharyngeal lumen (Au *et al.* 2009; Bandyopadhyay *et al.* 2009; Romney *et al.* 2011). Mutations in these metal transporters result in increased sensitivity to *Staphylococcus aureus* (Bandyopadhyay *et al.* 2009) and *Pseudomonas aeruginosa* (Rajan *et al.* 2019) infections, suggesting that manganese and/or iron sequestration from the luminal space may quell bacterial pathogenicity.

A study by Shapira *et al.* (2006) showed that *PDB1.1* is upregulated when infected by *P. aeruginosa*. Since PDB1.1 likely sequesters manganese in vacuoles near the apical surface of enterocytes, it may also play a role in limiting manganese availability for pathogens. Since *PDB1.1* is also upregulated by cadmium heavy metal toxicity (Huffman *et al.* 2004) it may be easily overlooked as a general stress response, however it has also been documented that transcription of CDFs can also be induced by other metals including cadmium (Cubillas *et al.* 2014). Future studies should examine *PDB1.1* mutants for increased susceptibility to pathogens, as well as determine if expression is also induced by another general stressor (such as heat shock), to discern whether it indeed plays a role in nutritional immunity.

4.5 *R02F11.3* null mutation does not lead to neuronal damage under basal conditions

High or chronic exposure to environmental manganese can lead to the development of Parkinson's disease or a similar condition of extrapyramidal dystonia called manganism (Quadri *et al.* 2012). Familial Parkinson-like disease can also develop as a result of mutations in SLC30A10, a manganese efflux transporter located in the liver and nervous system (Quadri *et al.* 2012; Tuschl *et al.* 2012). The link between manganese toxicity and neurological damage, specifically of the basal ganglia, led to further investigation of manganese-induced neurodegeneration in *C. elegans*. Two independent studies in the worm have confirmed that excess manganese damages dopaminergic neurons in Pdat-1::GFP marked strains, likely due to

increased production of reactive oxygen species (Settivari *et al.* 2009; Benedetto *et al.* 2010). Settivari *et al.* (2009) demonstrated that deletion or knock-down of divalent metal uptake transporter SMF-1, confers a neuroprotective effect after acute manganese exposure; this suggests that increased intracellular manganese may play a role in neurodegeneration. Conversely, loss of function mutations in manganese efflux transporter should then be more sensitive to manganese-induced oxidative stress.

Expression data indicates that manganese efflux transporter, R02F11.3, is also expressed in dopaminergic ciliated sensory neurons (Cao *et al.* 2017; Hutter and Suh 2016). We reasoned that null mutant R02F11.3 (*gk5490*) may accumulate high levels of manganese under basal conditions and therefore would be susceptible to neuronal damage, a consequence experienced by patients with SLC30A10 mutations (Quadri *et al.* 2012; Tuschl et a. 2012). Observations of Pdat-1::GFP-marked CEP neurons in a R02F11.3 null background showed no discernable difference from that of wildtype's. However, the extent of the damage may be too subtle under basal conditions and would require acute manganese stress to reveal its sensitivity. Alternatively, one can also monitor the basal slowing response (a behaviour governed by dopaminergic neurons) for defects.

4.6 Nematodes are robust against metal insults

We have identified 31 putative "nematode-specific" drug targets for anthelmintic development and chose to characterize two related genes (as well as their paralogs) in the genetically tractable model, *C. elegans*. These genes: *PDB1.1*, *F41C6.7*, *ZK185.5*, *K07G5.5*, *F56C9.3*, and *R02F11.3*, are members of the ubiquitous cation efflux transporter family and are likely responsible for the transit of metals out of the cytoplasm. Both PDB1.1 and R02F11.3 work to defend the worm from manganese toxicity through (presumably) metal sequestration in

the intestine and extrusion via the excretory system, respectively. K07G5.5 and ZK185.5 also seem to contribute to manganese detoxification, though their contributions are much smaller. While others detoxify, experimental results suggest that F41C6.7 distribute manganese to other cells or tissues. Although our results indicate PDB1.1, R02F11.3, ZK185.5, K07G5.5, and F41C6.7 are involved in maintaining manganese homeostasis, more experimental evidence is required to definitively demonstrate the proteins' role as manganese transporters. This can be achieved through CRISPR-Cas9 gene-edited Caco-2 human intestinal epithelial cells grown in transwells. Caco-2 transwells have been used to elucidate the modes and direction of solute transport in intestinal cells, including carrier-mediated transport (Hidalgo and Li 1996; Artursson, Palm, and Luthman 2012) and the movement of manganese (Leblondel and Allain 1999). Indeed a study by Scheiber *et al.* (2019) used Caco-2 transwells in the functional analysis of ZIP14, a manganese-selective SLC, and was able to validate its ability to transport manganese. Nevertheless, these first descriptions of manganese CDFs in *C. elegans* help broaden our understanding of metal homeostasis and may lend insight into the furtive lives of parasitic nematodes.

Although manganese homeostasis is critical for the health and survival of organisms, the abolishment of Mn-CDFs does not diminish the fitness of *C. elegans* under regular growth conditions. Calibrating the right concentration of metals at the right place and time would conceivably require multiple modalities of control (including expulsion via transporters, storage in vesicles, and chelation by metallothioneins) and/or redundancies in the system to ensure proper distribution. This may explain why worms with impaired or non-functional CDFs would develop or behave otherwise normally. In addition, parasites have been proposed as indicators of environmental pollution since the 1980's because they are able to concentrate heavy metals to a

detectible concentration (Sures *et al.* 1999; Morris *et al.* 2016; Sures *et al.* 2017). In fact, some parasitic helminths accumulate metal toxicants more readily than their hosts (Sures *et al.* 1999; Pascual and Abollo 2005) suggesting that they have developed a sophisticated line of defense against heavy metal poisoning. Given the robustness of *C. elegans* and parasitic helminths to environmental metals, and their likely multilateral approach to managing metal-induced stress, these genes are unlikely to be good candidates for anthelmintic drug development. However, this study has uncovered and characterized critical proteins in manganese detoxification and have generated a resource of genetic tools for further analysis of metal homeostasis in *C. elegans*.

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Appendices

Appendix A Bioinformatics

A.1 Gene ontology and InterPro ID search terms

Gene ontology annotation	Gene ontology term name
GO:0004672	Protein kinase activity
GO:0004930	G-protein coupled receptor activity
GO:0005247	Voltage-gated chloride channel activity
GO:0005267	Potassium channel activity
GO:0005272	Sodium channel activity
GO:0005216	Ion channel activity
GO:0005254	Chloride channel activity
GO:0005245	Voltage-gated calcium channel activity
GO:0055085	Transmembrane transport

InterPro ID	InterPro short description
IPR017452	GPCR_Rhodpsn_7TM
IPR019427	7TM_GPCR_serpentine_rcpt_Srw
IPR013099	K_chnl_dom
IPR014743	Cl-channel_core
IPR006201	Neur_channel
IPR001873	ENaC (epithelial sodium channel)
IPR000719	Prot_kinase_dom
IPR002524	Cation_efflux

Final terms were manually curated from dataset of 373 unique genes through search of key words: “GPCR”, “G-protein”, “kinase”, “channel”, “cation”, “transmembrane transport”. InterPro ID search terms also included all 130 InterPro domains from “The Druggable Genome” list of druggable targets (Table 1 of Hopkins and Groom 2002).

A.2 33 "nematode-specific" and druggable orthologs

Gene	CDS name	InterPro ID	InterPro Description	Gene ontology term ID	Gene ontology term description
air-1	*K07C11.2*	IPR000719	Protein kinase domain	GO:0004672	Protein kinase activity
C24G6.2	*C24G6.2*	IPR000719	Protein kinase domain	GO:0004672	Protein kinase activity
C38C3.4	*C38C3.4*	IPR000719	Protein kinase domain	GO:0004672	Protein kinase activity
C44C10.3	*C44C10.3*	N/Av	N/Av	GO:0055085	Transmembrane transport
C55B7.10	*C55B7.10*	IPR000719	Protein kinase domain	GO:0004672	Protein kinase activity
clh-1	*T27D12.2*	IPR014743	Cl channel core	GO:0005247	Voltage-gated Cl channel activity
daf-38	*Y105C5A.23*	IPR017452	GPCR, Rhodopsin-like, 7TM	GO:0004930	GPCR activity
del-10	*T28D9.7*	IPR001873	Epithelial Na channel	GO:0005272	Na channel activity
dkf-1	*W09C5.5*	IPR000719	Protein kinase domain	GO:0004672	Protein kinase activity
F40A3.7	*F40A3.7*	IPR017452	GPCR, Rhodopsin-like, 7TM	GO:0004930	GPCR activity
frpr-14	*K07E8.5*	IPR017452	GPCR, Rhodopsin-like, 7TM	GO:0004930	GPCR activity
inx-7	*K02B2.4*	N/Av	N/Av	GO:0055085	Transmembrane transport
K01A6.6	*K01A6.6*	IPR019427	7TM GPCR, serpentine receptor class w (Srw)	N/Av	N/Av
lgc-33	*Y55F3BR.4*	IPR006201	Neurotransmitter-gated ion-channel	GO:0005216	Ion channel activity
lgc-34	*T27A1.4*	IPR006201	Neurotransmitter-gated ion-channel	GO:0005254	Cl channel activity
npr-31	*T07F8.2*	IPR017452	GPCR, Rhodopsin-like, 7TM	GO:0004930	GPCR activity
R02F11.3	*R02F11.3*	IPR002524	Cation efflux protein	GO:0055085	Transmembrane transport
R07D5.2	*R07D5.2*	IPR017452	GPCR, Rhodopsin-like, 7TM	N/Av	N/Av
sma-6	*C32D5.2*	IPR000719	Protein kinase domain	GO:0004672	Protein kinase activity
snf-6	*M01G5.5*	N/Av	N/Av	GO:0055085	Transmembrane transport
stg-1	*C18D1.4*	N/Av	N/Av	GO:0005245	Voltage-gated Ca channel activity
T09B4.7	*T09B4.7*	IPR000719	Protein kinase domain	GO:0004672	Protein kinase activity
T12B3.2	*T12B3.2*	N/Av	N/Av	GO:0055085	Transmembrane transport
trpa-2	*M05B5.6*	N/Av	N/Av	GO:0005216	Ion channel activity
twk-1	*F21C3.1*	IPR013099	K channel domain	GO:0005267	K channel activity
twk-13	*R04F11.4*	IPR013099	K channel domain	GO:0005267	K channel activity
twk-26	*C33D12.3*	IPR013099	K channel domain	GO:0005267	K channel activity
twk-28	*C52B9.6*	IPR013099	K channel domain	GO:0005267	K channel activity
twk-40	*T28A8.1*	IPR013099	K channel domain	GO:0005267	K channel activity
twk-7	*F21C3.1*	IPR013099	K channel domain	GO:0005267	K channel activity
unc-58	*T06H11.1*	IPR013099	K channel domain	GO:0005267	K channel activity
ZK185.5	*ZK185.5*	IPR002524	Cation efflux protein	GO:0055085	Transmembrane transport
ZK596.2	*ZK596.2*	IPR000719	Protein kinase domain	GO:0004672	Protein kinase activity

CDS = CoDing Sequence

Appendix B *C. elegans* strains

B.1 Strains used and generated in this project

Strain	Allele	Description	Selection
VC4428	*gk5056*	*ZK185.5* CRISPR knock-out, 5x backcrossed	Pmyo-2::GFP (pharyngeal) & G418 resistant
VC4262	*gk5085*	*K07G5.5* CRISPR knock-out, 5x backcrossed	Pmyo-3::GFP (body wall muscle) & G418 resistant
VC4429	*gk5339*	*F56C9.3* CRISPR knock-out, 5x backcrossed	Pmyo-2::GFP (pharyngeal) & G418 resistant
VC4430	*gk5363*	*F41C6.7* CRISPR knock-out, 6x backcrossed	Pmyo-2::GFP (pharyngeal) & G418 resistant
VC4463	*gk5490*	*R02F11.3* CRISPR knock-out, 5x backcrossed	Pmyo-2::GFP (pharyngeal) & G418 resistant
VC4228	*gk5056; gk5085*	*ZK185.5* & *K07G5.5* CRISPR double knock-out	Pmyo-2::GFP (pharyngeal), pMyo-3::GFP (body wall muscle) & G418 resistant
VC4367	*gk5056; gk5085; gk5363*	*ZK185.5*, *K07G5.5*, & *F41C6.7* CRISPR triple knock-out	Pmyo-2::GFP (pharyngeal), pMyo-3::GFP (body wall muscle) & G418 resistant
VC4562	*gk5636*gk5339; egIs1*	*F56C9.3* CRISPR knock-out with marked Pdat-1::GFP marked dopaminergic neurons	Pdat-1::GFP
VC4563	*gk5637*gk5363; egIs1*	*F41C6.7* CRISPR knock-out with marked Pdat-1::GFP marked dopaminergic neurons	Pdat-1::GFP
VC4564	*gk5638*gk5490; egIs1*	*R02F11.3* CRISPR knock-out with marked Pdat-1::GFP marked dopaminergic neurons	Pdat-1::GFP
VC4581	*gk5652*gk5085; egIs1*	*K07G5.5* CRISPR knock-out with marked Pdat-1::GFP marked dopaminergic neurons	Pdat-1::GFP
VC4646	*gk5715*	*PDB1.1::mCherry*	N/A
VC4647	*gk5716*	*ZK185.5::GFP*	N/A
VC4648	*gk5717*	*F41C6.7::GFP*	N/A
VC4719[±]	*ok3114*	*PDB1.1* (*ok3114*), 4x backcrossed	N/A
BZ555[±]	*egIs1*	*Pdat-1::GFP*	Pdat-1::GFP

[±] Obtained through the *Caenorhabditis* Genetics Center

B.2 Genetic description of cation diffusion facilitator CRISPR-Cas9 deletion mutants

Gene	Allele	# of transcripts	Transcript used to design CRISPR deletion	# of exons	Comments
ZK185.5	*gk5056*	1	ZK185.5.1	7	Deletes all 7 exons
K07G5.5	*gk5085*	1	K07G5.5.1	5	Deletes exons 1 to most of 4
F41C6.7	*gk5363*	1	F41C6.7.1	13	Deletes all 13 exons
F56C9.3	*gk5339*	2	F56C9.3.1	6	Deletes all 6 exons. Both isoforms share the same exons.
R02F11.3	*gk5490*	2	R02F11.3b.1	6	Deletes exons 2 to most of 5 (to avoid deleting ncRNAs embedded within the intron)

B.3 Sequence validation primers

Primer Name	Primer Sequence (5' --> 3')
ZK185.5-1 SeqVal F	GCAGTTATCCCTGTCCTATTT
ZK185.5-1 SeqVal R	CTGAAAGTTCACCCGAAGTGTTATG
ZK185.5-1 WT F	GAGCTCGAGCCACGTTTATAG
ZK185.5-1 WT R	CACGTAGCATCGGGTCTTATC
K07G5.5-1 SeqVal F	ATGGTAACGCCGCTCTGATT
K07G5.5-1 SeqVal R	ATGATCCTCCATGCGCACAC
K07G5.5-1 WT F	TCCGGTAAAACCAGACGCTC
F56C9.3-1 SeqVal F	GTTTGTCAGTCTCGGTCGATAG
F56C9.3-1 SeqVal R	GGTAACACTGATTGGTGGAATG
F56C9.3-1 WT R	GTTGCCTCTATCTGCTCACTATC
F41C6.7-1 SeqVal F	TCGAATTCGTGTTGAATGTTGA
F41C6.7-1 SeqVal R	ACAATTCCATCACATTACCGTCC
F41C6.7-1 WT F	TCAGCCAAAGTTTTATTGCCGA
R02F11.3-1 SeqVal F	AAGAACTGTCGAAACGAGATTG
R02F11.3-1 SeqVal R	ATGTGTCAACGGAGTCTGCC
R02F11.3-1 WT F	TCGGTGAAGAACCCAAGCTC
NeoR F	TTCCTCGTGCTTTACGGTATCG
pMyo-2 R	CCCTCAATGTCTCTACTTGT
pMyo-3 R	TCAACCTGATCTTTGTTGCAGC

B.4 Sequence validation scheme for CRISPR deletion mutants

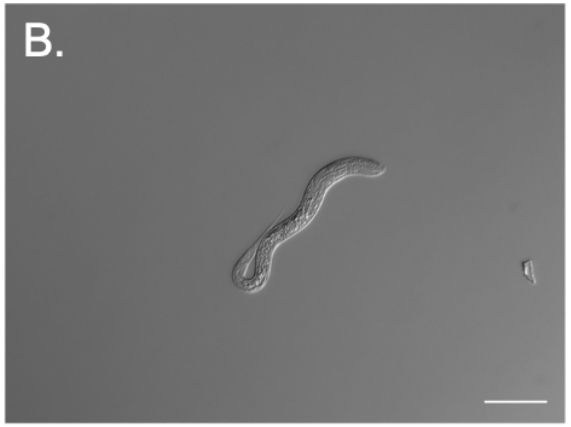
Gene	Allele	US Primer Pair	US Product Size	DS Primer Pair	DS Product Size	WT Check	WT Product Size (in N2)	Excision Check	Excised Product Size
ZK185.5	*gk5056*	ZK185.5-1 SV F pMyo-2 R	1982 bp	NeoR F ZK185.5-1 SV R	1561 bp	ZK185.5-1 WT F ZK185.5-1 WT R	1244 bp	ZK185.5-1 SV F ZK185.5-1 SV R	2549 bp
K07G5.5	*gk5085*	K07G5.5-1 SV F pMyo-3 R	1020 bp	NeoR F K07G5.5-1 SV R	1680 bp	K07G5.5-1 WT F K07G5.5-1 SV R	1010 bp	K07G5.5-1 SV F K07G5.5-1 SV R	1749 bp
F56C9.3	*gk5339*	F56C9.3-1 SV F pMyo-2 R	823 bp	NeoR F F56C9.3-1 SV R	1904 bp	F56C9.3-1 SV F F56C9.3-1 WT R	1398 bp	F56C9.3-1 SV F F56C9.3-1 SV R	1735 bp
F41C6.7	*gk5363*	F41C6.7-1 SV F pMyo-2 R	940 bp	NeoR F F41C6.7-1 SV R	1356 bp	F41C6.7-1 WT F F41C6.7-1 SV R	862 bp	F41C6.7-1 SV F F41C6.7-1 SV R	1302 bp
R02F11.3	*gk5490*	R02F11.3-1 SV F pMyo-2 R	1850 bp	NeoR F R02F11.3-1 SV R	1998 bp	R02F11.3-1 WT F R02F11.3-1 SV R	1348 bp	R02F11.3-1 SV F R02F11.3-1 SV R	2854 bp

US = upstream
DS = downstream

Sequence validation primers are designed to check the upstream and downstream junctions between genomic DNA and the inserted selection cassette. SeqVal F primers pair with pMyo-2 R primers. SeqValR primers pair with NeoR F primers. All upstream and downstream products should be present in the CRISPR/Cas9 deletion mutants. The wildtype product should be present in N2 controls and absent in the mutant strains.

Appendix C PDB1.1*(ok3114)* under high manganese condition

C.1 DIC images of PDB1.1(*ok3114*) under normal and high manganese conditions



PDB1.1(*ok3114*) mutants grown on NAMM plates with either (A) 0 µM supplementary manganese or (B) 500 µM supplemental manganese for 3 days.