The mechanism of the *peel-1 zeel-1* toxin-antidote system

Lews Caro

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Reading Committee:

Michael Ailion, Chair

Robert H. Waterston

Adam Steinbrenner

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University of Washington

Abstract

The mechanism of the *peel-1 zeel-1* toxin-antidote system

Lews Caro

Chair of the Supervisory Committee:
Michael Ailion
Department of Biochemistry

Part 1:

Toxin-antidote systems are selfish genetic elements composed of a linked toxin and antidote. The *peel-1 zeel-1* toxin-antidote system in *C. elegans* consists of a transmembrane toxin protein PEEL-1 which acts cell autonomously to kill cells. Here we investigate the molecular mechanism of PEEL-1 toxicity. We find that PEEL-1 requires a small membrane protein, PMPL-1, for toxicity. Together, PEEL-1 and PMPL-1 are sufficient for toxicity in a heterologous system, HEK293T cells, and cause cell swelling and increased cell permeability to monovalent cations. Using purified proteins, we show that PEEL-1 and PMPL-1 allow ion flux through lipid bilayers and generate currents which resemble ion channel gating. Our work shows that PEEL-1 kills cells by co-opting PMPL-1 and creating a cation channel.

Part 2:

Centromeric histones (CenH3s) are essential for chromosome inheritance during cell division in most eukaryotes. CenH3 genes have rapidly evolved and undergone repeated gene duplications

and diversification in many plant and animal species. In *Caenorhabditis* species, two independent duplications of *CenH3* (named *hcp-3* for *HoloCentric chromosome-binding Protein 3*) were previously identified in *C. elegans* and *C. remanei*. Using phylogenomic analyses in thirty-two *Caenorhabditis* species, we find strict retention of the ancestral *hcp-3* gene and ten independent duplications. Most *hcp-3L* (*hcp-3-like*) paralogs are only found in 1-2 species, are expressed in both males and females/ hermaphrodites, and encode histone fold domains with 69-100% identity to ancestral *hcp-3*. We identified novel N-terminal protein motifs, including putative kinetochore protein-interacting motifs and a potential separase cleavage site, which are well conserved across *Caenorhabditis* HCP-3 proteins. Other N-terminal motifs vary in their retention across paralogs or species, revealing potential sub-functionalization or functional loss following duplication. An N-terminal extension in the *hcp-3L* gene of *C. afra* revealed an unprecedented protein fusion, where *hcp-3L* fused to duplicated segments from *hcp-4* (nematode CENP-C). By extending our analyses beyond *CenH3*, we found gene duplications of six inner and outer kinetochore genes in *Caenorhabditis*, which appear to have been retained independent of *hcp-3* duplications. Our findings suggest that centromeric protein duplications occur frequently in *Caenorhabditis* nematodes, are selectively retained for short evolutionary periods, then degenerate or are lost entirely. We hypothesize that unique challenges associated with holocentricity in *Caenorhabditis* may lead to this rapid 'revolving door' of kinetochore protein paralogs.

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DEDICATION

This thesis is dedicated to my parents, Bertha Lucia Caro and Guillermo Alexander Caro.

Los quiero mucho.

Chapter 1. INTRODUCTION

A major goal of my graduate studies was to gain a mechanistic understanding of evolutionarily unique proteins. This led to my primary project, investigating the mechanism of a selfish toxin-antidote system from *C. elegans*: *peel-1 zeel-1*. Here, I focused on the mechanism of PEEL-1 toxicity. I found that PEEL-1 toxicity requires a second protein, PMPL-1. By co-expressing PEEL-1 and PMPL-1 in a heterologous system, I demonstrated that these two proteins are sufficient for toxicity and act by creating a novel cation leak channel. This work is contextualized in Section 1.1, where I discuss the current mechanistic understanding of several animal toxin-antidote systems. I present my findings on the PEEL-1 mechanism in Chapter 2. More preliminary data on the mechanism and evolution of *peel-1 zeel-1* are in Chapter 4.

My second project was on centromeric histones, an essential protein which counterintuitively undergoes rapid evolution. I characterized the evolution of centromeric histones in holocentric nematodes and found multiple, short-lived duplications of these proteins. I identified novel motifs in nematode centromeric histones that act as putative sites of protein-protein interactions. This work is presented in Chapter 3.

1.1 ANIMAL TOXIN-ANTIDOTE SYSTEMS AND THEIR MECHANISMS

"Natural selection", the process of favorable traits increasing in frequency over generations, is a pillar of evolutionary biology *(Darwin 1859)*. On the surface, this implies that only genes which encode these favorable traits can spread in populations. However, selfish genetic elements (SGEs) spread in populations by biasing their inheritance into progeny, even at

the expense of host fitness *(Lindholm et al., 2016, Werren 2011)*. SGEs come in many shapes and sizes. Some of the smallest SGEs are mobile genetic elements like transposons, which over replicate in genomes by copy-paste or cut-paste mechanisms *(McClintock 1950, Shapiro 1969)*. Larger SGEs such as centromeres can consist of repetitive, non-coding DNA that manipulates asymmetrical female meiosis to favor their inheritance *(Pardo-Manuel de Villena and Sapienza 2001, Henikoff et al., 2001, Melters et al., 2013, Lampson and Black 2018)*. Regardless of their size or mechanism, SGEs are pieces of DNA that spread in populations without necessarily providing a fitness benefit to hosts.

Toxin-antidote (TA) systems are an aggressive SGE that often guarantee their inheritance by killing non-inheriting progeny. TAs consist of two linked genes: a toxin expressed in the parent and an antidote expressed in the progeny *(Burga et al., 2020)*. The parental toxin is cytoplasmically transmitted to progeny; in animals, the toxin is inherited through the sperm or egg. This toxin causes death or developmental delay of progeny unless they inherit the TA, which provides the antidote *(Burga et al., 2020)*. The tight linkage between the toxin and antidote genes ensure that they are almost always inherited together, acting as one genetic element. Overall, TA elements selectively poison non-inheritors in order to increase their own representation in the next generation.

TA systems were first discovered in bacteria from so-called plasmid addiction systems. Here, a plasmid ensures its inheritance by encoding a stable toxin and a less-stable antidote. This differential stability is key for bacterial TAs. The toxin's stability allows its cytoplasmic inheritance while the antidote is quickly degraded *(Aizenman et al., 1996, Van-Melderen et al., 1996)*. This differential stability ensures that only daughter cells lacking the TA plasmid are affected by the toxin. Interestingly, bacterial TA systems are not exclusively present in plasmids.

Many bacterial TA systems exist within the chromosomal DNA. These genomic TA systems are not selfish but instead are important for cell persistence in unfavorable environments *(Hayes 2003, Fineran et al., 2009, Labrie et al., 2010)*. Mechanisms of toxicity from both plasmid and chromosomal TAs include inhibition of gyrase *(Bernard and Couturier et al., 1992; Jiang et al., 2002)*, mRNA cleavage *(Christensen et al, 2003; Zhang et al., 2003; Pedersen et al., 2003)*, and loss of membrane potential *(Wilmaerts et al., 2018)*. Meanwhile, antidotes can act by directly binding the toxin protein *(Dao-Thi et al., 2002, Madl et al., 2006, Kamada et al., 2003)* or inhibiting expression of the toxin *(Gerdes et al., 1992)*. Bacterial TAs are by far the best understood TA systems; much less is known about eukaryotic TA systems.

Many fungal TA systems act during meiosis to kill non-inheriting spores *(Bravo Núñez et al., 2018)*. Some *wtf* TAs from *Schizosaccharomyces pombe* encode two isoforms of the same gene *(Zanders et al., 2014, Hu et al., 2017, Nuckolls et al., 2017)*. The resulting toxin and antidote proteins are largely similar, but the antidote has an extended N-terminus *(Nuckolls et al., 2017)*. The mechanism of toxicity is unknown, but the toxin either aggregates or localizes to the Golgi *(Nuckolls et al., 2020, Zheng et al., 2023)*. The antidote can interact with the toxin, re-localizing the protein and preventing its toxicity *(Nuckolls et al., 2020, Zheng et al., 2023)*. There are many *wtf* genes in *Schizosaccharomyces* species, however, and it is not known whether they all work through the same mechanism. Other fungal TAs such as *spok (Grognet et al., 2014)* and *spk-1 (Turner and Perkins, 1979, Svedberg et al., 2021)* are also encoded by single genes, but how these systems work is unclear.

TA elements were once thought to be rare in animals, but this idea is beginning to be challenged. About a dozen animal TAs have been discovered *(Beeman et al. 1992, Seidel et al., 2008, Ben-David et al., 2017, Ben-David et al. 2021, Noble et al., 2021)*, and almost all of these

are in hermaphroditic species of *Caenorhabditis* nematodes. All known animal TA systems appear to be segregating within species, partly because the approaches used to identify new TAs require hybridization, biasing for these segregating TAs. Nevertheless, the fact that animal TAs naturally segregate within species demonstrates that TAs are a source of intraspecific hybrid sub-fertility and can therefore form genetic barriers within species.

Some of the best understood animal TAs are the Medea element in the red flour beetle, *peel-1 zeel-1* in *C. elegans*, *sup-35 pha-1* in *C. elegans*, and *slow-1 grow-1* in *C. tropicalis*. I summarize below the current mechanistic understanding of these TAs.

Medea element from T. castaneum

The Medea element (maternal-effect dominant embryonic arrest) from the red flour beetle, *Tribolium castaneum*, was the first TA system discovered in animals. Medea was found through hybrid crosses between beetle strains from the United States and Singapore *(Beeman et al. 1992)*. While hybrid F1 progeny developed normally, crossing these hybrids together resulted in lethality in the F2. In backcross experiments, high lethality was only seen when hybrid F1 females were crossed to US males, but not Singapore males. Furthermore, this lethality was not seen when hybrid males were backcrossed to either parent. These crosses indicated the presence of a dominant, maternal-effect genetic system that acted by "self-selection" *(Beeman et al. 1992)*. Despite being the first animal TA discovered, the molecular components of this TA system are not known. The candidate region for the Medea genes has been mapped to a 100kb region containing a Tc1 transposon *(Lorenzen et al., 2008)*, pseudogenes, and at least three intact protein-coding genes *(Lorenzen et al., 2008)*. Among these genes is a gene encoding a protein domain previously only found in bacteria *(Knizewski et al., 2007)*. Another is a gene homologous

to *blot*. In fruit flies, *blot* has both maternal and zygotic functions *(Lorenzen et al., 2008, Johnson et al., 1999)*, making it an interesting candidate gene for this TA.

sup-35 pha-1 from C. elegans

The *sup-35 pha-1* TA system consists of the SUP-35 toxin and the PHA-1 antidote. This TA has a unique story of discovery because PHA-1 was thought to be a master regulator of development for several decades *(Schnabel and Schnabel 1990)* before it was recognized as a TA element *(Ben-David et al., 2017)*. *pha-1* mutants generated in the lab were found to have pharyngeal defects that result in death, so *pha-1* was thought to regulate the development of the pharynx *(Schnabel and Schnabel 1990)* and other tissues *(Kuzmanov et al., 2014)*. However, a *C. elegans* strain from Hawaii (DL238) lacked this gene entirely, casting doubt on the essential role of *pha-1* in development. This inconsistency led to the discovery that PHA-1 was an antidote for a maternal toxin, SUP-35 *(Ben-David et al., 2017)*. The *sup-35 pha-1* genes shared expected features of a toxin-antidote system: the genes are tightly linked, the toxin is cytoplasmically inherited through the mother, and zygotic expression of the antidote prevents toxicity. The genetics of the *sup-35 pha*-1 TA system have been well-studied, and two genes are required for toxicity: *sup-36* and *sup-37 (Schnabel et al., 1991)*. However, the precise molecular functions of the toxin and antidote are not yet understood.

slow-1 grow-1 from C. tropicalis

The recently described *slow-1 grow-1* TA system from *C. tropicalis (Ben-David et al. 2021)*, adds a new layer of complexity to animal TAs. Expression of the *slow-1* toxin is controlled by imprinting, also known as a parent-of-origin effect *(Pilota et al., 2024)*. Maternal

inheritance of *slow-1 grow-1* elicited expected selfish activity, resulting in overrepresentation of *slow-1 grow-1* in the F2 generation. However, paternal inheritance of the TA caused absence of toxin activity in the next generation. In other words, the activity of the TA is dependent on the genotype of the grandparents, parents, and progeny. Both *slow-1* mRNA and SLOW-1 protein are maternally loaded into embryos, but the parent-of-origin effect is specifically attributed to the maternal mRNA *(Pilota et al., 2024)*. Progeny affected by SLOW-1 do not die but instead experience a developmental delay *(Ben-David et al. 2021)*. SLOW-1 is homologous to a nuclear hormone receptor and contains two predicted transmembrane domains. Meanwhile, GROW-1 is evolutionarily novel *(Ben-David et al. 2021)*. The mechanism of toxin and antidote activity are unknown.

peel-1 zeel-1 from C. elegans

The *peel-1 zeel-1* TA is currently the best characterized animal TA system and is also the only known TA to act through a paternal, sperm-delivered toxin. *peel-1* codes for a toxin and *zeel-1* codes for an antidote. This TA was discovered in hybrid crosses between the *C. elegans* N2 lab strain and a Hawaiian strain (CB4856) *(Seidel et al., 2008)*. When F1 hybrid males are backcrossed to CB4856 hermaphrodites, about half of the progeny arrest during development. However, backcrosses to N2 did not result in lethality. Furthermore, this phenotype was specific to hybrid males since backcrossing hermaphrodite hybrids to parental males resulted in minimal F2 lethality. This inheritance pattern fits a model of paternal, cytoplasmic inheritance of a toxin that requires a linked, zygotic antidote for viability. The antidote gene, *zeel-1*, was identified through transgenic rescue of antidote activity. It took three years to molecularly identify the toxin gene, *peel-1*, partly because it was unannotated in the genome. Natural *C. elegans* strains that

had antidote activity but lacked toxin activity allowed identification of the *peel-1* gene *(Seidel et al, 2011)*.

The *peel-1* gene codes for an evolutionarily novel, toxic protein. PEEL-1 is 174 amino-acids and predicted to contain four transmembrane domains. PEEL-1 is exclusively expressed in the male germline and is packaged in sperm-specific organelles (called fibrous body membranous organelles) by an N-terminal packaging sequence. After fertilization, PEEL-1 is presumably delivered to the zygote plasma membrane during fusion between the sperm and egg, but the actual location of PEEL-1 within embryos has not been determined. Toxicity from sperm-delivered PEEL-1 manifests in the 2-fold stage of developing embryos, with visible defects in the muscle, epidermis, and excretory cell *(Seidel et al., 2011)*. Presumably, sperm-delivered PEEL-1 ends up in these tissues since ectopic expression in each tissue causes similar phenotypes. Ectopic expression in adults causes several cellular phenotypes, including necrotic vacuoles, swollen cells, and disintegrated tissues *(Seidel et al., 2011)*. Ectopic expression also showed that PEEL-1 toxicity is cell-autonomous, meaning that toxicity occurs in the cell containing PEEL-1. So far, all adult somatic tissues appear to be susceptible to PEEL-1 toxicity. PEEL-1 may therefore disrupt some fundamental cellular process, but the precise mechanism of PEEL-1 toxicity is not known. Possible toxin mechanisms include inhibition of the sodium-potassium pump, pore formation, ion channel formation, or physical disruption of the plasma membrane.

The antidote, ZEEL-1, prevents toxicity from PEEL-1. ZEEL-1 is a predicted six-pass transmembrane protein, with a large soluble domain *(Seidel et al., 2011)*. The transmembrane region is lineage-specific, containing no homology outside of *C. elegans*. Meanwhile, the soluble domain is homologous to ZYG-11, a conserved substrate-recognition subunit of an E3 ubiquitin

ligase *(Seidel et al., 2011)*. As expected, *zeel-1* is expressed before PEEL-1 toxicity. Antidote activity is also tissue-specific; for example, ZEEL-1 expression in the epidermis prevents PEEL-1 toxicity in this specific tissue *(Seidel et al., 2011)*. This expression pattern and tissue-specific antidote activity leads to a model of ZEEL-1 interacting with PEEL-1, causing PEEL-1 ubiquitylation and degradation. However, this hypothesis has not been directly tested.

Other animal toxin-antidote systems

In addition to the TAs described above, other animal TAs have been studied including *msft-1* from *C. briggsae (Widen et al., 2023)*, *mll-1 smll-1* from *C. elegans (Zdraljevic et al., 2024)*, and unnamed TAs from *C. tropicalis (Ben-David et al. 2021, Noble et al., 2021)*. Investigations into animal TAs have shed light on other aspects of biology, including providing insight into possible origins of imprinting *(Pilota et al., 2024)*, horizontal gene transfer in eukaryotes *(Widen et al., 2023)*, and paternal contributions to embryonic development *(Seidel et al., 2011)*. Like chromosomal TA systems in bacteria that benefit hosts, animal TAs have possibly become domesticated by host genomes. Recent work suggests that *peel-1(+)* worms have a slight fitness advantage when competed against *peel-1(-)* worms in laboratory conditions *(Long et al., 2023)*. Feasibly, TA systems could arise and spread via their selfish activity and then become domesticated by hosts, eliciting some fitness benefits. Since some animal TAs code for evolutionarily novel proteins, TA element domestication might be a path for novel cellular functions, as seen in bacteria *(Hayes 2003, Fineran et al., 2009, Labrie et al., 2010)*. Therefore, Studying the mechanisms of animal TA systems can therefore provide insights into new biology.

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Chapter 2. TOXIN MECHANISM OF THE *PEEL-1 ZEEL-1* TOXIN-ANTIDOTE SYSTEM

The work in this chapter was done in collaboration with Aguan D. Wei (Seattle Children's Research Institute), Christopher A. Thomas (University of Washington), Galen Posch (University of Washington), Ahmet Uremis (University of Washington), Michaela C. Franzi (University of Washington), Sarah J. Abell (University of Washington), Andrew H. Laszlo (University of Washington), Jens H. Gundlach (University of Washington), and Jan-Marino Ramirez (Seattle Children's Research Institute).

2.1 INTRODUCTION

Selfish genetic elements ensure their inheritance, even at the expense of host fitness. Toxin-antidote (TA) systems are one class of selfish element, made up of genetically linked toxin and antidote genes. The toxin is cytoplasmically inherited across generations while the cognate antidote is expressed in progeny. In animal TA systems, the parental toxin is transmitted to progeny via the sperm or egg. Offspring that do not inherit the TA genetic element are affected by the toxin, typically resulting in death or developmental defects (*1*), whereas the offspring which inherit the TA genetic element express the cognate antidote to prevent toxicity. Therefore, TAs guarantee their presence in the next generation by killing non-inheriting offspring. Recent work suggests that TA elements are more common among animals than previously thought (*2*, *3*). However, the mechanisms of toxin and antidote activity remain largely unknown for animal TA systems.

One of the best characterized animal TA systems is *peel-1/zeel-1* in *C. elegans* (*4*). The *peel-1* toxin is expressed during sperm development but is not toxic to sperm. Mature sperm carry PEEL-1 protein and fertilization delivers the toxin, resulting in developmental arrest of embryos (*5*). However, offspring which inherit the TA element evade death by embryonic expression of the antidote, *zeel-1* (Fig. 2.1A). Expression of PEEL-1 in adult worms also causes death (*5*), suggesting toxicity is not specific to a developmental stage. Furthermore, PEEL-1 acts cell-autonomously in *C. elegans*; ectopic expression of PEEL-1 in specific tissues causes death of these cells, with no defects in neighboring cells (*5*). So far, no adult somatic cells have been found to be immune to PEEL-1, suggesting that toxicity works by disrupting a fundamental cellular process. In this study, we dissect the molecular mechanism of PEEL-1 toxicity. We find that PEEL-1 co-opts a conserved membrane protein of unknown function, PMPL-1. Together these proteins are sufficient to create a toxic, cation leak channel. Our findings therefore describe the molecular mechanism of toxicity of an animal toxin-antidote system.

2.2 RESULTS

2.2.1 PMPL-1 is required for PEEL-1 toxicity

To identify other genes required for PEEL-1 toxicity, we performed a large forward genetic screen for PEEL-1 suppressors in *C. elegans*. We mutagenized worms carrying transgenes for heat-shock inducible *peel-1* expression (*hsp-16.41p*::*peel-1*) (*5*). We isolated only two full-suppressors of heat-shock PEEL-1 toxicity, and both suppressors carry mutations in F47B7.1 (hereafter named *pmpl-1*) (Fig 2.1B). Endogenous, sperm-delivered PEEL-1 is also suppressed by *pmpl-1* mutants (Fig. 2.1C), but not via a paternal-effect (fig. 2.S1), suggesting that PMPL-1 acts in embryos to facilitate PEEL-1 toxicity and does not act in sperm. *pmpl-1*

codes for a 59 amino acid protein predicted to be an integral membrane protein (Fig. 2.1D). The *pmpl-1* mutants were identified as a missense allele (*yak52*, A47T) and a full-gene deletion allele (*yak103*) (see Materials and Methods).

The *pmpl-1* expression pattern is consistent with its role in PEEL-1 toxicity. The *pmpl-1* promoter drives expression in embryos prior to toxicity from sperm-delivered PEEL-1 (fig. 2.S2A) (*5*). *pmpl-1* is also widely expressed in several adult tissues (fig. 2.S2B), consistent with heat-shock PEEL-1 toxicity in adults (*5*). Publicly available RNA-seq data (*6*) indicates that *pmpl-1* expression levels are lowest in the male gonad compared to all other tissues, consistent with wild-type sperm being unaffected by PEEL-1 (fig. 2.S2C). These data suggest that PMPL-1 is required for cell susceptibility to PEEL-1 toxicity. We further confirmed that co-expression of PEEL-1 and PMPL-1 in the vulval muscle cells of *pmpl-1* mutants caused specific toxicity in this tissue (Fig. 2.1E and fig. 2.S2D). Defects were not observed in surrounding tissues, indicating that PEEL-1 and PMPL-1 act in the same cell to cause toxicity.

2.2.2 PMPL-1 is a conserved membrane protein of unknown function

PMPL-1 belongs to the Plasma Membrane Proteolipid 3 (PMP3) family of proteins, so we named it "PMP3-Like protein 1." PMP3 proteins are widely present in bacteria, plants, and fungi, and are found in some simple animals (*7*). The role of PMP3 proteins in animals is unknown, but PMP3 proteins in plants, fungi, and bacteria are important for cold-stress resistance, membrane protein trafficking, and ion homeostasis (*8–11*). *pmpl-1* mutant worms do not have any obvious phenotypes. However, PMPL-1 is highly conserved among nematodes (fig. 2.S3). We identified 15 PMP3-like proteins in *C. elegans* (fig. 2.S4) through BLAST searches. PMPL-2 (also known as TXT-9) is most similar to PMPL-1 (75% similarity) and was found in an

RNAi screen for defective transcellular chaperone signaling (*12*). However, there is no known molecular function for any *C. elegans* PMP3-like protein.

PMP3 proteins are thought to contain two transmembrane spanning domains (*13*, *14*), consistent with DeepTMHMM predictions for PMPL-1 (fig. 2.S5A) (*15*). However, AlphaFold2 predicts PMPL-1 as a monotopic protein, with four helices passing through only one leaflet of a lipid bilayer (Fig. 2.1D and fig. 2.S5B) (*16*, *17*). We favor the monotopic prediction of PMPL-1 because two recently solved structures of a bacterial photosynthetic complex showed a PMP3 protein within the complex having a similar monotopic structure (*18*, *19*).

In contrast to *pmpl-1*'s broad conservation in nematodes (fig. 2.S3), *peel-1* is found only in *C. elegans* and has no homology to any known protein (*5*). The dramatic difference in conservation of these genes suggests that PMPL-1 has biological roles other than supporting PEEL-1 toxicity. *pmpl-1* is on a different chromosome than *peel-1 zeel-1* (see Materials and Methods), making it genetically unlinked from the TA element. This suggests that PEEL-1 co-opts PMPL-1 for its own use.

2.2.3 PEEL-1 and PMPL-1 are sufficient for toxicity in HEK293T cells

Given that *pmpl-1* was the only full suppressor of PEEL-1 toxicity found in our screen, we hypothesized that PEEL-1 and PMPL-1 may be the only two components required for toxicity. We expressed these proteins in human embryonic kidney cells (HEK293T) and assayed for cytotoxicity, using lactate dehydrogenase (LDH) release into the culture media as a measure of plasma membrane rupture (*20*). We found that each protein alone was not toxic, but co-expression of PEEL-1 and PMPL-1 resulted in significant cytotoxicity (Fig. 2.2A). Other PMP3-

like proteins such as *C. elegans* PMPL-2 and yeast PMP3 were not toxic with PEEL-1 (Fig. 2.2B), suggesting that PMPL-1 has a specific role in toxicity that is not universal to the PMP3 family. The reconstitution of toxin activity in a heterologous system suggests that PEEL-1 and PMPL-1 are sufficient for toxicity and may act by disrupting an essential, conserved cellular process. Antidote activity could also be reconstituted in HEK293T cells by co-expression of ZEEL-1, resulting in reduced toxicity (Fig. 2.2A).

2.2.4 Plasma membrane localization of PEEL-1 and PMPL-1 is critical for toxicity

PEEL-1 and PMPL-1 localize to several membrane-bound compartments in HEK293T cells and *C. elegans*. Both proteins localize to the endoplasmic reticulum (ER), while PMPL-1 also localizes to the plasma membrane (PM) and to lipid droplets (Fig. 2.2C-D and fig. 2.S6A), consistent with the predicted monotopic topology. Although PEEL-1::eGFP is not easily detectable on the PM of transfected HEK293T cells, stable expression of PEEL-1::eGFP in both HEK293 cells and in *pmpl-1* knock-out worms shows clear PM localization (fig. 2.S6B-C), suggesting that PMPL-1 is not required for PEEL-1 to reach the plasma membrane.

To determine in which compartment these proteins perform their toxic roles, we prevented their movement to the PM by the addition of a GBR1 ER-retention tag (*21*). ER-retention tags on either protein resulted in more than an 80% drop in toxicity, and retention tags on both proteins completely suppressed toxicity (Fig. 2.2E), suggesting that PEEL-1 and PMPL-1 do not act in the ER and likely perform their toxic roles at the PM.

2.2.5 PEEL-1 toxicity causes cell swelling in HEK293T cells

LDH release is an end-point measure of plasma membrane rupture, but it was unclear whether plasma membrane rupture was a primary or secondary effect of toxicity. Imaging 48 hours after transfection showed that 92% of HEK293T cells co-expressing PEEL-1 and PMPL-1 were swollen, about double their typical size (Fig. 2.3A-B). Live imaging showed cells exhibiting abnormal phenotypes approximately 16 hours after transfection. Cells began with slight swelling of the nucleus and cell, followed by jetting out round protrusions of 5-10 μm, similar in size to the nucleus (Fig. 2.3C). These large protrusions are short-lived, typically existing for less than 10 minutes before being reabsorbed by the cell. Eventually, cells swell evenly in all directions (Fig. 2.3D). Swollen cells often lose the integrity of their plasma membrane, followed by ER fragmentation and ER swelling (fig. 2.S7). By 48 hours after transfection, we observe various outcomes for swollen cells: cell lysis, cell detachment from the plate, and swollen, intact cells attached to the plate. Our LDH assay likely captures only the first of these phenotypes and may underestimate the fraction of cells experiencing toxicity. The cell swelling phenotype is reminiscent of the necrotic vacuoles previously described in PEEL-1 affected embryos in *C. elegans* (*5*).

The cell swelling phenotype caused by PEEL-1 toxicity suggests that cell death is non-apoptotic (*22*). Indeed, *C. elegans* mutants defective in apoptosis (*ced-3*) and apoptotic cell engulfment (*ced-2* and *ced-5*) are still susceptible to PEEL-1 toxicity (fig. 2.S8) (*23–25*). Other mammalian cell death pathways such as pyroptosis and necroptosis appear to be absent from *C. elegans* (*26*). This suggests that the cytotoxic phenotypes we observe are a direct effect of PEEL-1 and PMPL-1 activity rather than indirect induction of programmed cell death.

2.2.6 PEEL-1 has an amphipathic helix that is critical for toxicity

PEEL-1 has no homology to known proteins (*5*), so we turned to structural predictions to identify important regions in PEEL-1. AlphaFold2 predicted a low-confidence structure with six alpha-helices (Fig. 2.4A) (*16*, *17*). The longest four helices matched DeepTMHMM's prediction of four transmembrane domains (fig. 2.S5C). Closer inspection revealed that the fourth predicted transmembrane domain is amphipathic (Fig. 2.4B). An amphipathic helix (AH) has opposing hydrophilic and hydrophobic faces and is a critical feature of known pore-forming toxins. For example, actinoporins are a diverse family of toxins which oligomerize their AHs to construct channels (*27*). The properties of the predicted PEEL-1 AH are similar to actinoporin AHs. The PEEL-1 AH is 22 amino acids in length (residues 111-132), making it long enough to span the lipid bilayer (*28*). The hydrophobic moment (μH), a measure of the degree of amphipathicity, is within the range of actinoporin channel-forming toxins (fig. 2.S9) (*29*). One notable difference is that the PEEL-1 AH is more hydrophobic than actinoporin AHs (fig. 2.S9), suggesting that the PEEL-1 AH may always exist within the membrane, unlike in actinoporins which have soluble AH conformations.

We hypothesized that the PEEL-1 AH may perform a similar role as it does in actinoporins, where AHs construct the lining of a toxic channel. To test this, we determined whether this helix was required for toxicity in HEK293T cells and *C. elegans*. We found that deleting the last two helices of PEEL-1 did not severely impair toxicity ("-28 aa" and "-39aa" in Fig. 2.4C-D). However, deleting the AH resulted in complete loss of toxicity in both mammalian cells and worms ("-65 aa" in Fig. 2.4C-D). By deleting one amino acid at a time, we determined that toxicity in HEK293T cells was eliminated after removing at least 40 amino acids (fig. 2.S10). This PEEL-1(-40aa) mutant contains only three residues following the AH, possibly destabilizing the AH.

We also tested whether the amphipathic property of the PEEL-1 AH was critical for toxicity by creating a series of missense mutants that modified the AH's hydrophobic moment. Six single-missense mutants were tested. Three of these mutants attenuated PEEL-1 toxicity (S124F, L122Q, L115Q) while three mutants were still fully toxic (L118Q, S124V, L126Q) (Fig. 2.4E). However, all pairwise combinations of these three fully toxic mutants resulted in complete loss of toxicity (Fig. 2.4E). Across the nine missense mutants we tested, toxicity correlated well with the amphipathicity of the AH (Fig. 2.4F). These data suggest that the amphipathic property of the PEEL-1 amphipathic helix is critical for toxicity, supporting its potential role as the lining of a channel. However, we cannot rule-out alternative explanations for the importance of the AH, including roles in protein-protein interaction or protein stability.

2.2.7 PEEL-1 and PMPL-1 create a non-specific monovalent cation channel

We hypothesized that PEEL-1 toxicity was caused by disruption of ionic gradients across the cell membrane, leading to osmotic imbalance and subsequent cell swelling. This could be through two mechanisms: an ion leak channel, where specific ions flow down their electrochemical gradient, or a non-selective pore, causing flux of all ions and smaller osmolytes across the plasma membrane. To test these possibilities, we performed whole-cell patch-clamp electrophysiology on HEK293 cells in physiological ion conditions (high internal K^+, high external Na^+). Currents from cells transfected with *peel-1* alone or *pmpl-1* appeared similar to currents from untransfected cells (Fig. 2.5A and fig. 2.S11A-B). Obtaining recordings of cells co-transfected with *peel-1* and *pmpl-1* was difficult, due to variable timing of toxicity, and was complicated by loss of cells due to swelling. Therefore, we generated tetracycline-inducible cell lines that more tightly regulate PEEL-1 and PMPL-1 expression. We generated a HEK293 cell

line with tetracycline-inducible PMPL-1::mCherry and then introduced stable expression of either eGFP ("Control" cell line) or PEEL-1::eGFP ("Experimental" cell line). Cells with and without tetracycline are hereafter referred to as induced and uninduced, respectively. Nearly all Experimental cells swell 24 hours after induction (fig. 2.S12A), with the first signs of swelling occurring 6 hours after induction (fig. 2.S12B).

We performed whole-cell patch clamp recordings of uninduced cells, and at 6 hours and 28 hours after induction (Fig. 2.5B-C). Similar to untransfected naïve HEK293 cells, uninduced Control cells exhibited small instantaneous negative (inward) currents at negative potentials without leak subtraction, and these currents did not increase after induction (Fig. 2.5B and fig. 2.S11C). In contrast, uninduced Experimental cells exhibited a larger instantaneous inward current compared to uninduced Control cells and naïve HEK293 cells, and the magnitude of these currents further increased after 28 hours of induction (Fig. 2.5C and fig. 2.S11C). When clamped at the most negative potential (-100 mV), inward currents from uninduced and 28-hour induced Experimental cells were 7-fold and 30-fold greater than naïve HEK293 cells, respectively. These data suggest that co-expression of PEEL-1 and PMPL-1 creates a constitutively active ionic channel.

The inward currents observed in uninduced Experimental cells (Fig. 2.5C and fig. 2.S11C) were instantaneous, exhibiting no voltage-dependent gating kinetics, consistent with a leak channel constitutively open at physiological resting membrane potentials. This ion leakage in uninduced Experimental cells was surprising since these cells appear healthy and do not have obvious growth defects. We detected some PMPL-1 expression in uninduced Control and Experimental cells (Fig. 2.5D and fig. 2.S12A), consistent with previous reports of leaky background expression from tetracycline-inducible promoters (*30–32*). Therefore, we reasoned

that uninduced Experimental cells may have sufficient background PMPL-1 expression to display an electrophysiological phenotype but not enough to cause cell swelling or cell death.

To characterize the ionic selectivity of channels formed by PEEL-1 and PMPL-1 co-expression, we performed ionic substitution recordings. We took advantage of the inward currents generated by uninduced Experimental cells since these cells are morphologically normal, reducing the possibility of contamination by endogenous HEK293 channels secondarily activated by cell swelling or cell death such as the LRRC8-associated volume-regulated anion channels (VRACs) (*33*, *34*). We substituted the physiological mixed cation bath solution (high Na^+/low K^+) with equivalent bath solutions containing single cations (Na^+, K^+, Cs^+, $NMDG^+$ or a combination of 20mM Ca^{2+}/120mM $NMDG^+$) and monitored instantaneous inward currents at negative potentials. We found that bath solutions with Na^+, K^+, or Cs^+ still yielded large inward currents, while bath solutions with $NMDG^+$ and Ca^{2+} abolished all inward currents (Fig. 2.5E and fig. 2.S13). These results suggest that PEEL-1 and PMPL-1 co-expression creates a channel that is permeable to monovalent cations (Na^+, K^+, and Cs^+) but impermeant to the divalent Ca^{2+} cation and the bulkier cation $NMDG^+$. We next tested for Cl^- permeability by recording with high internal Cl^- (140 mM KCl) and low external Cl^- (140mM $NMDG^+$). Any Cl^- permeability at negative potentials would cause Cl^- movement out of the cell and be recorded as a negative (inward) current. We observed no inward currents (Fig. 2.5E and fig. 2.S13), indicating that the channel is impermeable to Cl^-. Altogether, these results suggest that PEEL-1 and PMPL-1 create an ion channel rather than a non-selective pore. The PEEL-1 and PMPL-1 channel conducts monovalent cations and is impermeable to anions and divalent cations. An increase in PMPL-1 expression in induced Experimental cells may result in excessive influx of Na^+, sufficient to

overwhelm compensatory volume regulatory mechanisms, leading to osmotic dysregulation, cell swelling, and cell death.

2.2.8 Predicted PEEL-1 pentamer structure has features of an ion channel

Ion channels are typically made up of oligomeric complexes, so we used AlphaFold2 to determine possible oligomeric structures of PEEL-1. Structural predictions were of low-confidence due to PEEL-1's lack of homology to known proteins (*5*). Nevertheless, the predicted PEEL-1 pentamer has a striking resemblance to known cation channels (fig. 2.S14A-D): (a) the outside surface of the complex is hydrophobic, consistent with its location in lipid bilayers, (b) the complex has a central, uninterrupted hydrophilic pore, providing a potential path for ions through the complex, and (c) the opening of the pore region is surrounded by a ring of negatively charged residues. These features are strikingly similar to the structure of the ZAR1 cation channel, which is a toxic pentameric channel with a ring of acidic residues at the extracellular mouth of the pore that serve as a cation selectivity filter (*35*). In the predicted PEEL-1 pentamer, a similar ring of negative charges is formed by five D109 residues. Mutating this residue to an alanine (D109A) resulted in complete loss of PEEL-1 toxicity (fig. 2.S14E). This residue is two amino acids before the PEEL-1 AH. Five copies of the PEEL-1 AH meet in the middle of the complex, forming the pore-like region of the predicted structure (fig. 2.S14A-B), consistent with our model of PEEL-1 AHs constructing the lining of a channel.

2.2.9 In vitro reconstitution of the PEEL-1/PMPL-1 ion channel

We used an independent approach to test if PEEL-1 and PMPL-1 create an ion channel by assaying whether these purified proteins allow ions to flow through artificial planar lipid bilayers (Fig. 2.5F). PEEL-1 and PMPL-1 were individually expressed in *E. coli* with maltose-binding protein (MBP) tags, affinity purified in detergent, and incorporated into liposomes for delivery to planar lipid bilayers (fig. 2.S15 and Materials and Methods). Interestingly, PEEL-1 appears to be toxic to *E. coli* and removal of the PEEL-1 amphipathic helix attenuated this toxicity (fig. 2.S16). Truncation products of PEEL-1 were purified along with the full-length protein (fig. 2.S15). Only after adding PEEL-1 liposomes and PMPL-1 liposomes to the same bilayer did we observe discrete unitary conductance events similar to those created by typical ion channels (Fig. 2.5G and fig. 2.S17) (*35–38*). There was variation in single channel conductance and open duration (Fig. 2.5G and fig. 2.S17). Such variability could reflect heterogenous stoichiometries, or it could be due to technical limitations such as misfolded proteins, truncated PEEL-1 proteins in the sample, mixed orientation of proteins in the bilayer, or imprecise amounts of each protein in the bilayer. Nevertheless, the stepwise conductance *in vitro* and cation-selective property in cells suggest that PEEL-1 and PMPL-1 together construct a monovalent cation channel which causes osmotic dysregulation and cell death.

2.3 DISCUSSION

Our study provides unprecedented understanding of an animal TA system. We provide evidence that the PEEL-1 toxin co-opts a conserved membrane protein to create a cation channel which ultimately causes cell swelling and death. We show that PEEL-1 may be the primary structural component of the channel and hypothesize that PMPL-1 is required to gate the channel open. PEEL-1 may have evolved this co-option mechanism for temporal control over ion channel

activity, to avoid toxicity in sperm and early embryos. Prevention of off-target toxicity in the germline and before the maternal-to-zygotic transcriptional switch is likely an evolutionary hurdle faced by many animal TA systems. Therefore, co-option may be a common evolutionary route for toxicity in animal TA systems.

2.4 MATERIALS AND METHODS

2.4.1 Worm strains and maintenance

Worm strains were maintained using standard procedures (*39*). Strains used in this study are provided in Table 2.S1.

2.4.2 Isolation of suppressors of heat-shock induced PEEL-1

Worm strains XZ1047 and XZ1372 were mutagenized using ENU or EMS (*39*, *40*). These strains contained two copies of *hsp-16.41p*::*peel-1* in order to avoid isolating suppressors that have mutations in the transgene. F2 populations of worms were heat-shocked at 34°C for 2 hours and allowed to recover at room temperature overnight. Surviving worms were isolated. Approximately 200,000 haploid genomes were screened. *yak52* was isolated from EMS mutagenesis of XZ1047, while *yak103* was isolated from ENU mutagenesis of XZ1372.

We isolated a total of six independent suppressors of heat-shock PEEL-1 toxicity. Four of the mutations were partial suppressors while two were full suppressors. The causative genes were identified using genetic mapping and whole-genome sequencing. The four partial suppressors have mutations in mRNA export factors *nxf-1* and *nxt-1*, but act by affecting expression of *hsp-16.41p::peel-1* and do not suppress toxicity of endogenous, sperm-delivered PEEL-1 (*41*).

The two full suppressors *yak52* and *yak103* are recessive suppressors of PEEL-1 toxicity and have no other obvious phenotype. They were determined to be allelic by complementation testing and were mapped to the X chromosome by crossing to strains carrying visible or fluorescent markers on each chromosome (strains EG1000, EG1020, EG8040, EG8041). The closest linkage seen was to the visible marker *lon-2*.

2.4.3 Identification of pmpl-1

Strains XZ1177 and XZ1307 carrying 4X and 5X outcrossed pmpl-1(yak52) and pmpl-1(yak103) were subjected to whole-genome sequencing. DNA was purified according to the Hobert laboratory protocol (http://hobertlab.org/whole-genome-sequencing/). Illumina (XZ1177) or Ion Torrent sequencing (XZ1307) was performed at the University of Utah DNA Sequencing Core Facility. The dataset for XZ1177 contained approximately 17,000,000 reads of a mean read length of 36 bases, resulting in ~6X average coverage of the C. elegans genome. The dataset for XZ1307 contained approximately 12,800,000 reads of a mean read length of 147 bp, resulting in ~19X average coverage. The sequencing data were processed on the Galaxy server at usegalaxy.org (42). SNPs and indels were identified and annotated using the Unified Genotyper and SnpEff tools (43, 44). Although we found genes on the X chromosome in each strain containing nonsynonymous mutations, none of these genes were affected independently in both strains. Instead, to identify potential large deletion, we used BEDtools Genome Coverage tool to calculate sequencing coverage across the genome in intervals of consecutive bases with the same coverage (45). Intervals with zero sequencing coverage were then annotated using SnpEff, filtered to intervals on the X chromosome which overlapped protein-coding regions, and sorted by length. In XZ1307, the largest of these intervals on the X chromosome was found near where

yak103 and yak52 had been mapped and was subsequently confirmed to correspond to yak103, a 323-bp deletion spanning the F47B7.1 gene, from its 5'UTR to 3'UTR and deleting all the coding sequence. In XZ1177, we found two small regions of F47B7.1 lacking coverage in our whole genome sequencing. Via Sanger sequencing, we identified yak52 as a nonsynonymous mutation in one of these regions, with a G to A mutation that results in an A47T substitution.

2.4.4 Heat-shock PEEL-1 toxicity assay

Heat-shock PEEL-1 assays were performed with 50 gravid adults of each strain for each biological replicate. Adults were picked to clean NGM plates seeded with OP50 and heat-shocked (2 hours at 34°C). After recovering at room temperature for 2 hours, moving worms were counted. Non-moving worms were tested for response to touch stimulus and scored as dead if they did not move.

2.4.5 Sperm-delivered PEEL-1 toxicity assay

Sperm-delivered PEEL-1 toxicity in wild-type or *pmpl-1* mutant backgrounds was assayed by counting unhatched embryos. To make *pmpl-1*; *peel-1(+) zeel-1(+) / peel-1(-) zeel-1(-)* worms, AFS216 *peel-1(-) zeel-1(-)* males were crossed to *peel-1(+) zeel-1(+)* worms with *pmpl-1* mutations *yak103* (XZ2194), *yak52* (XZ2283), or wild-type *pmpl-1* (N2, control). F1 males were backcrossed to the parent strain (XZ2194, XZ2283, or N2) to obtain the desired genotype.

Embryonic lethality was assayed on individual, self-fertilizing hermaphrodites. Single worms were allowed to lay embryos for 16-24 hours before being removed from the plate. Plates were left for one more day to allow embryos to develop and hatch, and the progeny were scored.

Unhatched embryos and larval worms were counted. Unhatched embryos were scored as dead. Progeny were genotyped in bulk for *zeel-1(-)* by PCR using oGP65 (5'attctggagttcgtgaggtgc3') and oGP66 (5'ccctcctttcccacccaac3'). Only plates showing *zeel-1(-)* alleles were considered to have parents with the desired genotype, heterozygous *peel-1(+) zeel-1(+) / peel-1(-) zeel-1(-)*.

2.4.6 Plasmid construction

All plasmids used in this study are provided in Table 2.S2. Plasmids used for mammalian cell transfection were generated using Gibson assembly (*46*) using N1 vector backbone (CMV promoter). Constructs for tetracycline-inducible expression were cloned into pFTSH vector (gift from Nancy Maizels). Plasmids used for *C. elegans* transgenics were constructed using either Gibson assembly or Gateway cloning (Invitrogen). Typical Gateway cloning was performed using a 3-fragment approach (promoter, coding region, and fluorophore-UTR or UTR) into a destination vector, pCFJ150. Restriction digest was used to confirm the plasmid was correct. Sanger sequencing was used to confirm that constructs did not contain mutations (Table S2). Transgenic worms were made using microinjection. For each transgenic strain, we isolated at least two independent lines and confirmed similar results.

2.4.7 Microscopy

All microscopy was performed on a NikonTi2-E Crest X-light V2 spinning-disk confocal microscope. For live-cell imaging, mammalian cells were maintained in a humidified, heated chamber (37°C) in 5% carbon dioxide. For long-term imaging, the objective heater was also heated to 37°C. For imaging *C. elegans*, worms or embryos were picked onto a 2% agar pad,

immobilized in a humidified chamber for 10 minutes in 33mM sodium azide, and then immediately imaged.

2.4.8 Mammalian cell maintenance and generation of clonal cell lines

All mammalian cells were grown at 37°C and 5% carbon dioxide in DMEM with GlutaMAX (Thermo Fisher Scientific), 10% fetal bovine serum (RMBIO or Gibco), and 100 U/mL of penicillin-streptomycin (Gibco). All transfection-based cytotoxicity experiments were done in HEK293T cells (gift from Mary Claire-King). For electrophysiology experiments, HEK293 cells (CRL-1573; ATCC) were used. Tetracycline-inducible cells (tetON cells) were made in HEK293 Flp-In TReX cells (Invitrogen) (gift from Nancy Maizels). tetON cells were grown in media made with Tet system approved fetal bovine serum (Gibco).

Stable tetON lines were generated by co-transfection of FlpO (pOG44; Life Technologies) and PtetON::*pmpl-1*::mCherry (pLC79, in pFTSH backbone, a gift from Nancy Maizels). Two days after transfection, cells were plated at low density in 10cm plates with hygromycin B (150µg/mL). Two clonal populations were picked into media with hygromycin B (150µg/mL), grown to 80% confluency, and frozen in 10% DMSO at -80°C. One clonal population was used to generate the experimental and control cell line.

Experimental tetON and control tetON cell lines were generated parallel. One tetON::*pmpl-1*::mCherry clonal cell line was transfected with the respective plasmid, *peel-1*::eGFP (pGP9) or eGFP. Two days after transfection, cells were plated at low density in selective media containing both hygromycin B (150µg/mL) and G418 (400µg/mL). After about two weeks, multiple single colonies were picked and continued to be grown under selection. For the experimental cell line, more than half the picked clones which grew in selective media did

not have visible green fluorescence, presumably due to leaky *pmpl-1*::mCherry imposing selection against *peel-1*::eGFP-expressing cells. Therefore, only clones which had green fluorescence were maintained. This issue was not encountered during the generation of the control cell line. All experimental and control cell lines were screened following tetracycline treatment. 48 hours after tetracycline treatment, all cell lines had red fluorescence, all experimental cell lines had very few adhered cells (n= ~6), and all control cell lines had no obvious cellular phenotypes (n= ~10).

2.4.9 Cytotoxicity assay

Cytotoxicity in mammalian cells was measured using a colorimetric assay for LDH release (Promega CytoTox 96). 12-well plates were seeded with approximately $7x10^4$ HEK293T cells about 24 hours prior to transfection. Each well was transfected with a mixture of 1.5µg DNA and 3µg PEI in 200µL OptiMEM (Gibco). Combinations of three constructs were used (0.5µg each), using fluorophore-encoding vectors (mCherry or eGFP) as controls when less than three constructs are being tested. Supernatant was collected 43-45 hours after transfection and used in 96-well plates for LDH assays, following manufacturer protocol. Two technical replicates were measured for each well and absorption at 490nm was averaged between replicates. All experiments included wells for a non-killing control (fluorophore-encoding vectors), *peel-1*::eGFP alone, and *peel-1*::eGFP with *pmpl-1*::mCherry. The fold-change over *peel-1*::eGFP was calculated from transfections performed on the same day.

2.4.10 SDS-PAGE and western blots

For lysis of mammalian cells, cells were collected from 12-well plates and washed with PBS. Cell pellets were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (25mM Tris pH 7.4, 150mM NaCl, 0.1% SDS, 1% NP-40, 1% sodium deoxycholate) with DNase and Halt

Protease Inhibitor Cocktail (Thermo Fisher Scientific). After lysis (15min on ice), the sample was centrifuged (21,000 x *g*, 10min,4°C), and the supernatant was used for SDS-PAGE.

Following separation by SDS-PAGE, proteins were transferred onto a nitrocellulose membrane. After blocking in Intercept Blocking Buffer (LI-COR, 1hr room temperature) membranes were incubated with primary antibody and nutated overnight at 4°C. Mouse monoclonal anti-mCherry (a gift from Jihong Bai and the Fred Hutch Cancer Center antibody development shared resource center; 1:1000), mouse monoclonal anti-MBP (New England Biolabs, E8032; 1:10,000), rabbit polyclonal anti-GAPDH (Sigma Aldrich, G9545; 1:5,000). Appropriate secondary antibodies were used, either goat anti-mouse or donkey anti-rabbit conjugated to Alexa 680 or Alexa 790 (Invitrogen). Membranes were imaged on an Odyssey CLx (LI-COR Biosciences).

2.4.11 Electrophysiology

For electrophysiology on acutely transfected cells, plasmid constructs were transfected into HEK293 cells using Viafect reagent (E4981; Promega), following the manufacture's protocol. Cells were first prepared for transfection by plating onto 12-well tissue culture plates (Nunc 12-565-321; Thermo Fisher Scientific) at a density of ~0.5-2 x10^5 cells per well and grown to ~80-90% confluence with standard media, allowing for one confluent well per transfection condition. On the day of transfection, media were replaced with 0.5 mL fresh DMEM with 10% FCS, without P/S. Lipophilic/DNA transfection complexes were generated for each well, combining a total of ~1.0µg of plasmid DNAs with serum-free OptiMEM (Gibco 31985062) to a final volume of 100µL, then adding 3.0 µL Viafect with gentle trituration, allowing the mixture to assemble at 24°C for 30 minutes, and then added to each well, dropwise.

Transfected cells were incubated overnight at 37°C and visually monitored for transfection efficiency *in situ* using an inverted plate microscope equipped with fluorescence (Invitrogen EVOS M7000; Thermo Fisher Scientific). Transfection efficiencies were typically >70-80%. Specific amounts of plasmid DNAs (pDNAs) for acute transfections per well: a) pCMV::*peel-1*::eGFP (0.8 µg), b) pCMV::*pmpl-1*::mCherry (0.1µg) and pcDNA3 (0.8µg), c) pCMV::*peel-1*::eGFP (0.8 µg) and pCMV::*pmpl-1*::mCherry (0.8µg). Untransfected HEK293 cells served as controls.

Following overnight incubation, cells in transfected wells were dissociated with TrypLE (Gibco); and replated at low density onto 12 mm poly-*D*-lysine-coated glass coverslips (GG-12-pdl; NeuVitro) in 24-well tissue culture plates (FisherBrand FB012929; Thermo Fisher Scientific), for patch-clamp electrophysiology. Typically, ~10,000-15,000 cells were replated per well at sufficiently low density to isolated individual cells. This was necessary to prevent the formation of electrical junctions between contacting cells, which could preclude adequate space-clamp recording conditions. Recordings were performed from 0.5-3 days after replating at low densities. Control and Experimental stable HEK293 cell lines were similarly replated at low density on coverslips for patch-clamp recordings.

For patch-clamp recordings, coverslips containing adherent cells were transferred to a Zeiss AxoExaminer.A1 microscope, equipped with an 40X water immersion objective and epifluorescence capability. Pipettes were positioned with a Sutter MPC-325 micromanipulator (Novato). Whole-cell voltage-clamp recordings were acquired with an AxoClamp200B amplifier (Molecular Devices), using pClamp10.4. Composition of recording solutions are listed below:

Bath Solutions:

i. HEK293 bath (4 K^+, 145 Na^+) (in mM): 4.0 KCl, 145 NaCl, 2.0 $CaCl_2$, 2.0 $MgSO_4$, 10 HEPES, 10 glucose, pH to 7.4 with NaOH.

ii. *140 Na^+ (in mM):* 140 NaCl, 2.0 $CaCl_2$, 2.0 $MgCl_2$, 10 HEPES, pH to 7.4 with NaOH.

iii. *140 K^+ (in mM):* 140 KCl, 2.0 $CaCl_2$, 2.0 $MgCl_2$, 10 HEPES, pH to 7.4 with KOH.

iv. *140 Cs^+ (in mM):* 140 CsCl, 2.0 $CaCl_2$, 2.0 $MgCl_2$, 10 HEPES, pH to 7.4 with CsOH.

v. *140 $NMDG^+$ (in mM):* 140 N-methyl-*D*-glucamine ($NMDG^+$), 2.0 $CaCl_2$, 2.0 $MgCl_2$, 10 HEPES, pH to 7.4 with HCl.

vi. *20 Ca^{2+} (in mM):* 20 $CaCl_2$, 120 $NMDG^+$, 2.0 $MgCl_2$, 10 HEPES, pH to 7.4 with HCl.

Internal Pipette Solutions:

i. *140 K^+, low Cl^- (in mM)*: 140 K-*D*-gluconate, 1.0 $CaCl_2$, 2.0 $MgCl_2$, 10 HEPES, 2.4 EGTA, 4.0 Na_2ATP, 0.3 Na_2GTP, pH to 7.4 with KOH.

ii. *140 K^+, high Cl^- (in mM)*: 140 KCl, 1.0 $CaCl_2$, 2.0 $MgCl_2$, 10 HEPES, 2.4 EGTA, 4.0 Na_2ATP, 0.3 Na_2GTP, pH to 7.4 with KOH.

For all recordings of cationic currents, different bath solutions were used in combination with *140 K^+, low Cl^-* internal pipette solution. For recordings of Cl^- currents, *140 $NMDG^+$* bath solution was used in combination with *140 K^+, high Cl^-* internal pipette solution; under these conditions outward Cl^- conductance at negative potentials would be seen as inward currents by standard electrophysiological recording convention. Patch pipettes were pulled from borosilicate glass (1B120F-4; World Precision Instruments) on a P-97 Sutter Instruments puller (Novato), with resistances of 3.0-5.0 MΩ. Currents were allowed 3-5 mins to stabilize after achieving

whole-cell recording configuration, filtered at 5 kHz, and acquired at 10 kHz. Series resistance compensation was >80% for all recordings.

Mean currents normalized to cell capacitance were analyzed and plotted using pClamp10.4 (Molecular Devices) and Microsoft Excel. Leak subtraction correction was not applied to any of the data. Current-voltage (IV) data were plotted as means with standard errors (SE) and statistical calculations were performed in Prism (GraphPad).

2.4.12 Protein purification

MBP::PEEL-1::His_8 and MBP::PMPL-1 were purified from *E. coli* BL21(DE3) and C43(DE3) cells, respectively. An overnight culture (37°C, 220rpm, 100µg/mL ampicillin, 33µg/mL chloramphenicol) was used to inoculate 1L of LB media (100µg/mL ampicillin, 220rpm, baffled flask), grown at 37°C to OD_{600} of 0.4-0.7, and induced with IPTG (0.5mM). PMPL-1 cells were chilled on ice prior to induction, and then induced in an 18°C shaker overnight. PEEL-1 cells were induced at 37°C for 1 hour since longer induction at lower temperatures resulted in decreased yields. Cells were pelleted and either stored at -80°C or used immediately for purification.

For purification, cell pellets were resuspended in Buffer A (HEPES pH 7.4, 150mM NaCl, 5mM 2-mercaptoethanol, 10% glycerol). Cells were lysed using a Dounce homogenizer, followed by 30 minutes rocking at room temperature after addition of DNase, lysozyme, protease cocktail inhibitor (Pierce), and n-octyl-β-glucopyranoside detergent (β-OG, 2% final concentration) (Anatrace). Lysate was clarified by centrifugation (20 min, 20,000 x *g*, 4°C) before batch binding with amylose resin for 1-2 hours at 4°C. Resin was pelleted (5 min, 700 x *g*) and resuspended in wash buffer to load into a clean column. Four washes (5 column volumes) and four elutions (two column volumes) were performed by gravity flow. Wash buffer and

elution buffer contained 1% β-OG in Buffer A. Maltose (10mM) was included in elution buffer. Protein yield was estimated by absorbance at 260nm. Proteins were stored at 4°C for less than three days before incorporation into liposomes.

2.4.13 Proteo-liposomes

Liposomes containing MBP::PEEL-1::His_8 or MBP::PMPL-1 were made with 50% DOPC:POPS (1,2-dioleoyl-sn-slycero-3-phosphocholine: 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine). Lipid stocks were purchased in chloroform (Avanti Polar Lipids) and β-OG stock was made in methanol. Lipid films were made by mixing lipids with β-OG detergent (lipid:detergent molar ratio of 4:35) in glass vials, dried under a nitrogen stream (10-20 min), and further dried in a Speedvac evaporator (4-6 hours). Buffer A was added to the dried lipid-detergent mixture and resuspended via two or three rounds of bath sonication (5 min, room-temperature) with nutation (20 min, 4°C). Protein was added in an approximate protein:lipid molar ratio of 1:400 (PMPL-1) or 1:2,500 (PEEL-1). Detergent was removed by dialysis: 500µL of sample was dialyzed (20 kDa cutoff) in 250mL Buffer A with 0.5g Biobeads SM-2 (Bio-Rad) for 16-18 hours at 4°C. Dialyzed sample was floated through a density gradient (Histodenz 35%, 25%, 0%; Sigma-Aldrich) using ultracentrifugation (SW60Ti, 55krpm, 4°C, 30 min). Liposomes were collected from the top fraction (200µL, using a wide-bore pipette tip), aliquoted, flash frozen in liquid nitrogen, and kept at -80°C. Liposomes were thawed fresh on the day of each experiment. A second liposome prep was made from an independent protein purification with two adjustments: soybean lipids (Avanti Polar Lipids) with cholesterol were used for liposomes instead of DOPC and POPS, and 1mM EDTA was included in Buffer A.

2.4.14 Synthetic planar lipid bilayers

Two ~50µL wells (*cis* and *trans*) linked by a ~20µm PTFE aperture were filled with Buffer B (20mM HEPES, pH 7.4, 150mM NaCl, 10% glycerol, 1mM $CaCl_2$). A mixture of asolectin lipid (Sigma-Aldrich) and hexadecane was painted across the aperture to establish a planar bilayer membrane as previously described (*47*). To promote liposome fusions, an osmotic gradient across the bilayer was established by perfusion of Buffer C (20mM HEPES, pH 7.4, 600mM NaCl, 3.36% glycerol, 1mM CaCl2) into the *cis* well. A voltage of -180mV was applied across the membrane by two Ag/AgCl electrodes and the ion current was measured. Liposomes were added to the *cis* well and mixed via pipetting. Sharp transient spikes in currents were interpreted as successful liposome fusions. After observing channel activity Buffer D was perfused into the *cis* chamber (20mM HEPES, pH 7.4, 600mM NaCl, 10% glycerol) to remove free liposomes and a voltage of +180mV was applied since channel activity was more stable at +180mV than -180mV. Ion current was recorded at 50 kHz and downsampled to 50 Hz for analysis. The recorded ion current is divided by the applied voltage to give units of conductance. All-points histograms of conductance were constructed using a bin width of 2 pS and normalized based on probability density (area under the histogram equals 1).

We observed channels in 8 independent experiments and with two independent protein preps. A subset of experiments was run with controls, where we observed ion channels in 2 out of 3 trials after adding both PEEL-1 liposomes and PMPL-1 liposomes, but we did not see channel activity when adding PEEL-1 alone (n=4) nor PMPL-1 alone (n=3). A typical experiment ran as follows: 2uL of PEEL-1 liposomes and 2uL of PMPL-1 liposomes were added to the *cis* chamber and mixed via pipetting. Liposome fusions to the bilayer were confirmed by transient spikes in conductance, beginning approximately 5-20 minutes after addition and channel activity was seen approximately 5-60 minutes after fusions began. The amount of time

until channels were observed was used as a benchmark for control experiments, where 2uL of either PEEL-1 liposomes or PMPL-1 liposomes were added to the chamber and allowed to fuse for the same amount of time or longer than experimental runs.

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2.6 FIGURES

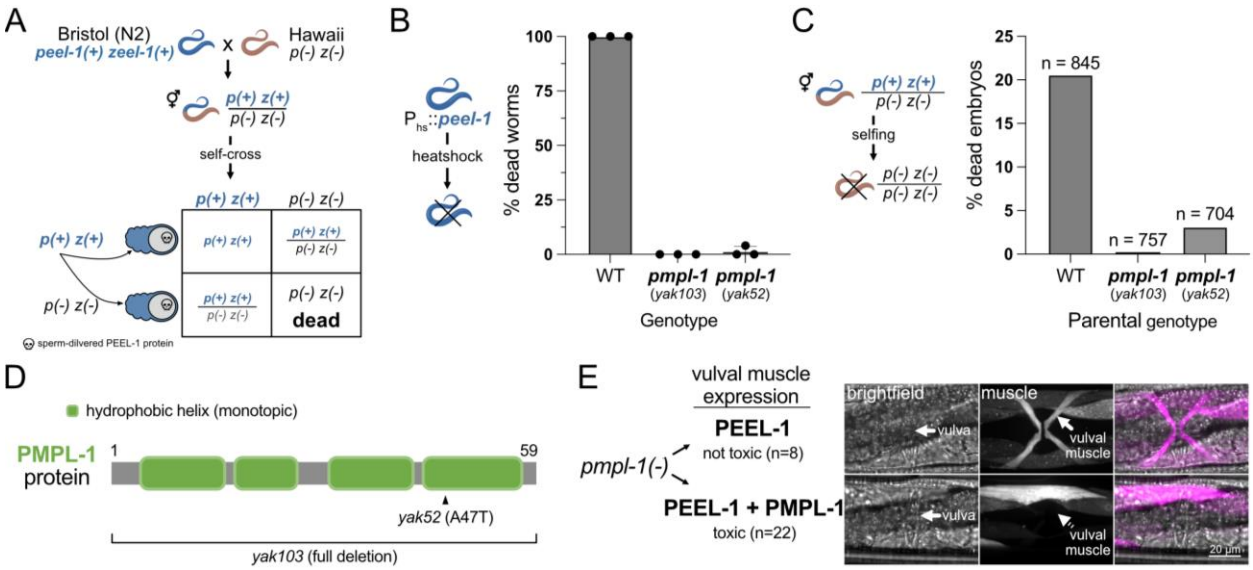

Fig. 2.1. PMPL-1 is necessary for PEEL-1 toxicity in *C. elegans*.

(A) Selfish activity of the *peel-1 zeel-1* toxin-antidote system in *C. elegans* shown in a genetic cross of strains from Bristol (which have the genetic element) and Hawaii (which lack the genetic element). Hermaphrodite worms heterozygous for the presence of *peel-1 zeel-1 (p(+) z(+) / p(-) z(-))* have 25% inviable progeny. This is due to sperm-delivered PEEL-1 toxicity causing developmental arrest of *zeel-1(-)* progeny. **(B)** Proportion of worms dying from ectopic, heat shock-PEEL-1 expression. Two *pmpl-1* mutant alleles (*yak103* and *yak52*) provide resistance to toxicity. **(C)** Proportion of dead, arrested embryos from self-fertilizing hermaphrodites heterozygous for *peel-1 zeel-1*. n = total progeny scored. **(D)** Predicted domain structure of the PMPL-1 protein with mutant alleles shown. The hydrophobic helices are predicted to be monotopic, passing through one leaflet of a lipid bilayer. **(E)** Body wall muscle (magenta) of *pmpl-1*(*yak103*) worms with vulval muscle-specific expression of PEEL-1 alone (top) or PEEL-1 and PMPL-1 (bottom). Vulval muscles appears normal with PEEL-1 alone but

are missing or atrophied when PEEL-1 and PMPL-1 are co-expressed in these cells. All worms expressing PEEL-1 and PMPL-1 in vulval muscle cells had missing or severely deformed vulval muscles (n=22). *peel-1* and *pmpl-1* are both GFP tagged. All channels are shown in fig. 2.S2D. Scale bar = 20μm.

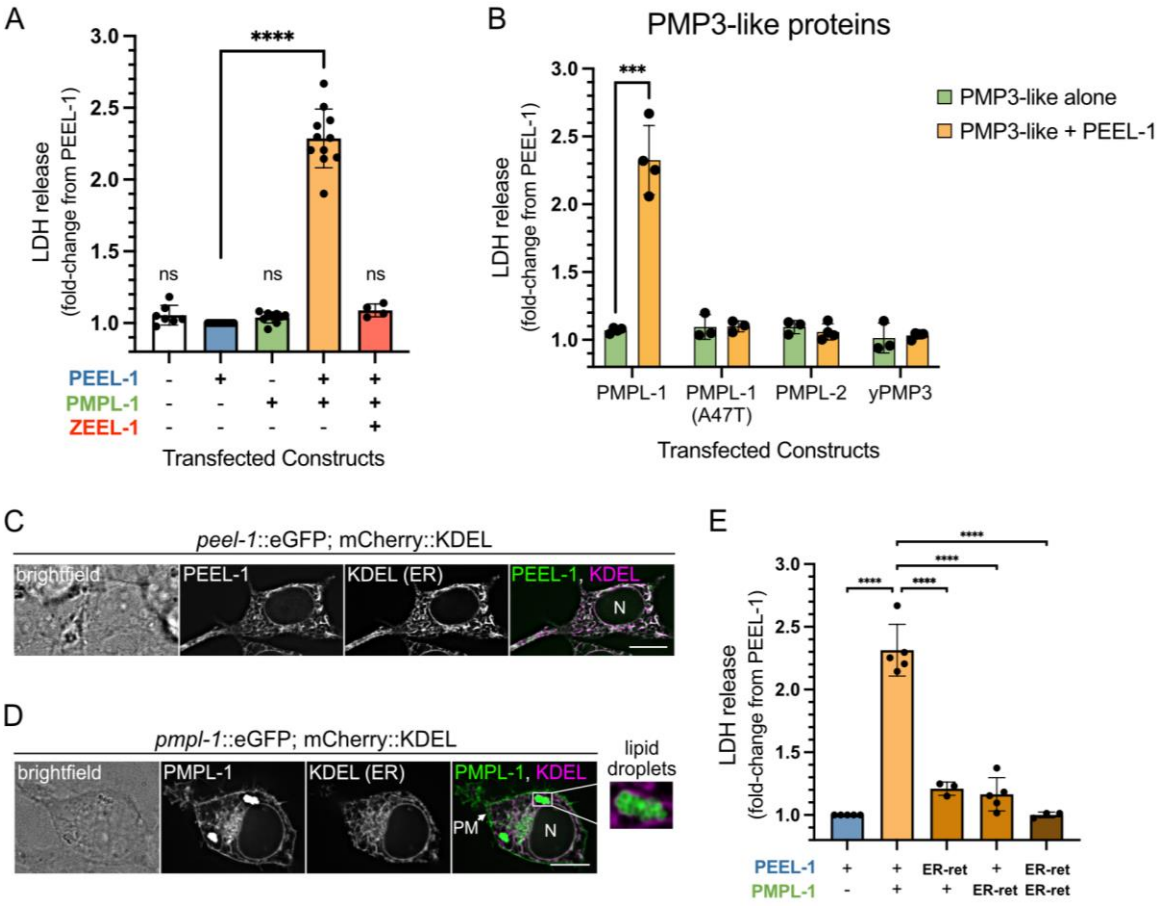

Fig. 2.2. PEEL-1 and PMPL-1 are sufficient for toxicity in HEK293T cells.

(A) Cytotoxicity (measured by LDH release) of combinations of three constructs transfected in HEK293T cells. Each data point is a biological replicate, normalized to LDH release from transfection with *peel-1*::eGFP in the same experiment. All plots show means with SD. Transfections combine constructs encoding for mCherry or eGFP (-) or a fluorescent-tagged protein (+): PEEL-1::eGFP (top), PMPL-1::mCherry (middle), or mCherry::ZEEL-1 (bottom). **(B)** Cytotoxicity of PMP3-like proteins alone or with PEEL-1::eGFP. The PMPL-1 *yak52* (A47T) mutant protein, *C. elegans* PMPL-2, and the yeast homolog yPMP3 are shown. **(C)** Live-cell imaging of a single cell transfected with an ER-marker (mCherry::KDEL) and *peel-1*::eGFP or **(D)** *pmpl-1*::eGFP. The cell nucleus is indicated (N). PMPL-1 is also seen on the plasma

membrane (PM) and on lipid droplets (inset). Scale bar = 10µm. **(E)** Cytotoxicity is suppressed by addition of the GBR1, ER-retention tag on the C-terminus of PEEL-1::eGFP or PMPL-1::mCherry. P-values in (A) and (E) calculated using one-way ANOVA with Dunnett's multiple comparisons test, comparing all samples to PEEL-1 alone in (A) and all samples to PEEL-1 with PMPL-1 in (E). In (B), multiple unpaired t-tests were used with Holm-Šídák test, comparing each PMP3-like protein alone to PMP3-like with PEEL-1 (***, $p < 0.001$; ****, $p < 0.0001$).

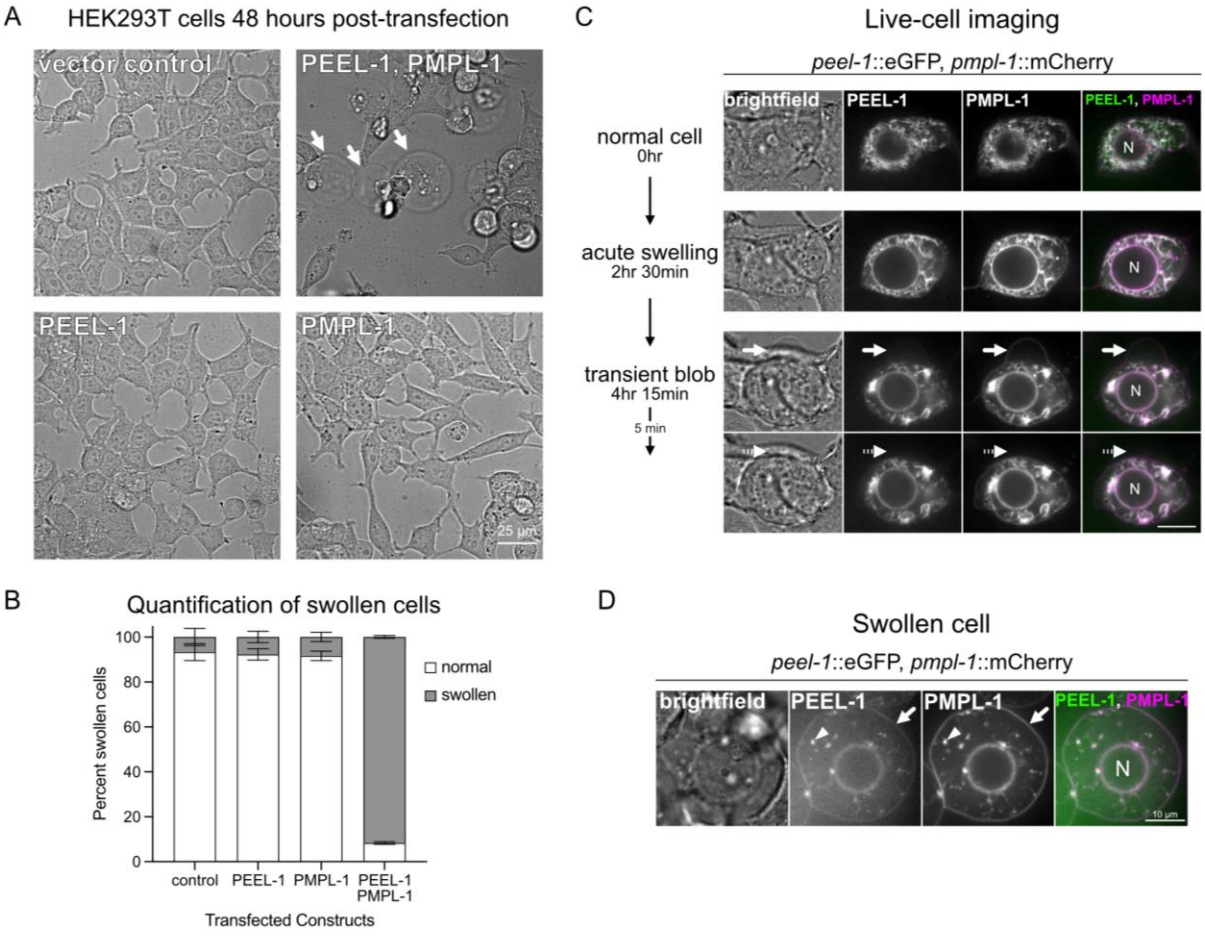

Fig. 2.3. PEEL-1 toxicity results in HEK293T cell swelling.

(A) Brightfield images of cells transfected with indicated constructs. Cells were imaged 48 hours after transfection. Scale bar = 25µm. Arrows point to swollen, transfected cells. **(B)** Percent of transfected cells that appear normal or swollen under brightfield. Mean with SD of three biological replicates is shown. 100 cells were scored for each condition in each biological replicate. **(C)** Selected frames of a live-cell imaging time-course experiment. A single cell is shown, co-expressing PEEL-1::eGFP and PMPL-1::mCherry. The nucleus is labeled ("N"). t=0 is 16 hours after transfection. Acute swelling can be seen (t=2hr 30min), followed by a transient blob or protrusion (arrow) jetted out by the cell (t=4hr 15min) and later reabsorbed (dotted arrow) (t=4hr 20min). Scale bar = 10µm. **(D)** A typical phenotype from a cell transfected with

PEEL-1::eGFP and PMPL-1::mCherry at 48 hours post-transfection. PEEL-1 and PMPL-1 can be seen on the plasma membrane (arrow) and in fragmented ER (arrowhead). Scale bar = 10μm.

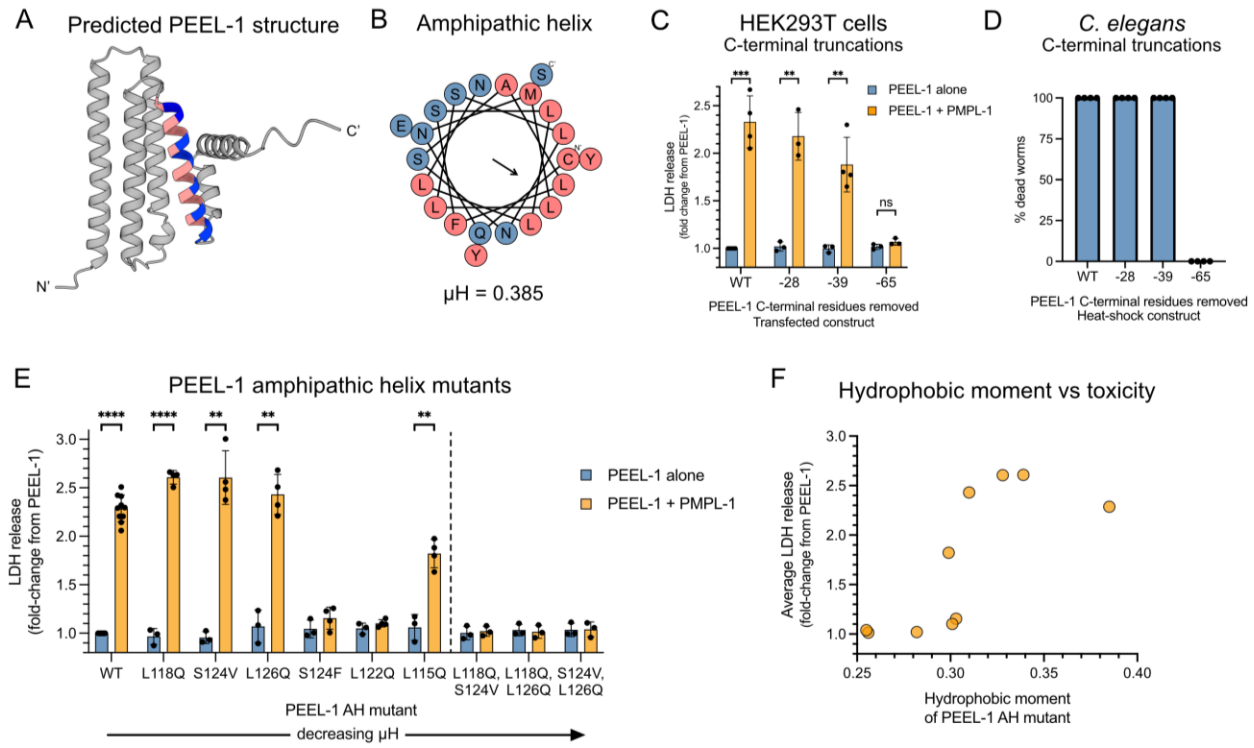

Fig. 2.4. PEEL-1's amphipathic helix is critical for toxicity.

(A) The AlphaFold2 predicted structure of PEEL-1 and **(B)** a helical wheel representation of the putative PEEL-1 amphipathic helix. Amphipathic helix residues are colored (pink = hydrophobic, blue = hydrophilic). **(C)** Cytotoxicity of a series of PEEL-1 C-terminal truncations expressed in HEK293T cells. The number of amino acids removed are indicated (ex. "-28" means the last 28 residues were removed). Each truncation removes an additional alpha helix. The "-65" truncation removes the amphipathic helix. **(D)** Percent dead worms after heat-shock PEEL-1 expression of the indicated truncation mutant. 50 worms were assayed for each data point, and two independent transgenic lines were tested or each construct. **(E)** Cytotoxicity of PEEL1 amphipathic helix missense mutants in HEK293T cells. Mutants are ordered by descending hydrophobic moment (μH). Six single mutants (left of dotted line) and three double mutants are shown (right of dotted line). All bar graphs show mean with SD. Statistics were

performed using multiple unpaired t-tests with Holm-Šídák test, comparing each PEEL-1 alone to PEEL-1 and PMPL-1 (**, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). **(F)** The average toxicity of missense mutants from (E) plotted against their hydrophobic moment.

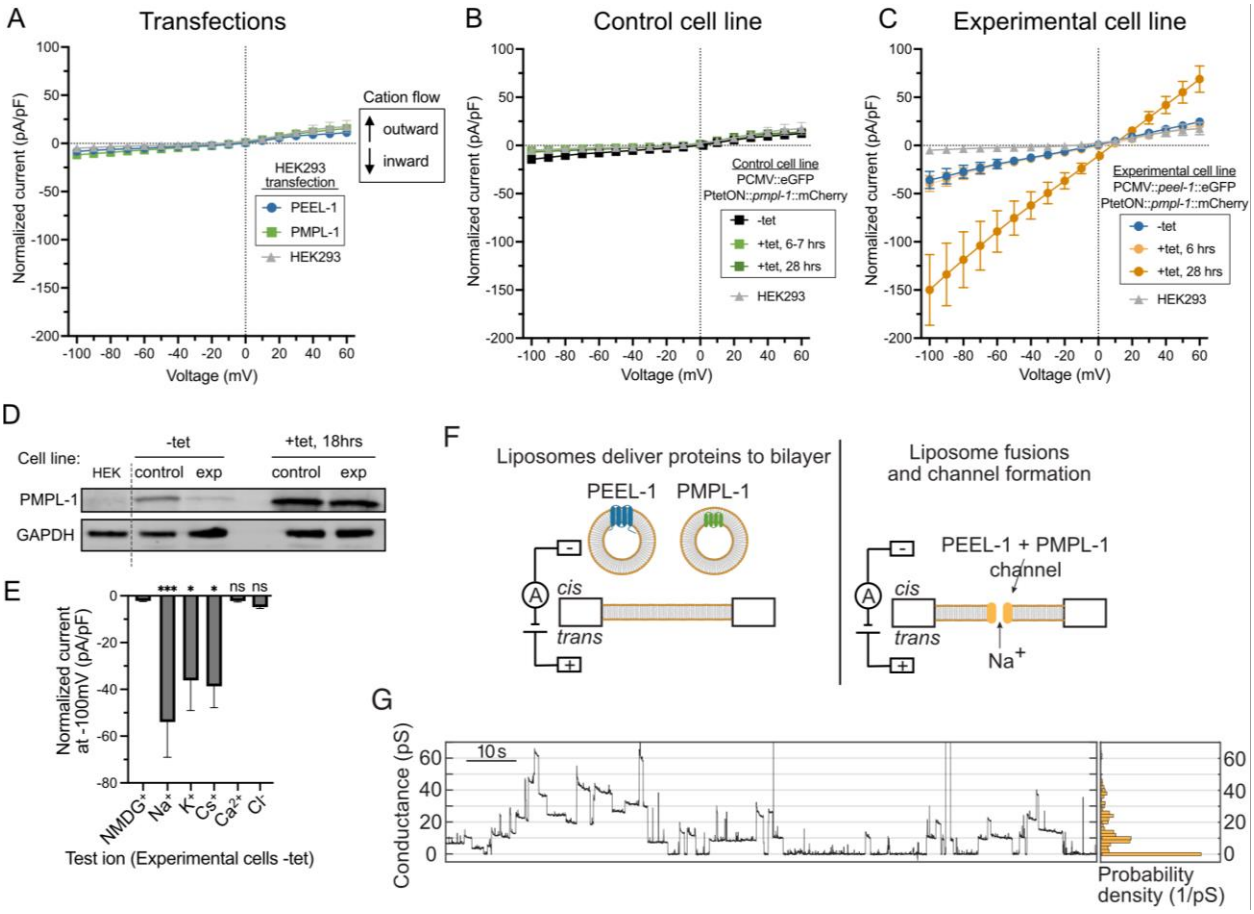

Fig. 2.5. PEEL-1 and PMPL-1 create a monovalent cation channel.

Current-voltage plots of whole-cell patch-clamp electrophysiology on **(A)** transfected cells, **(B)** Control cell line, and **(C)** Experimental cell line. High intracellular potassium (140mM K^+/ 8.6mM Na^+) and high extracellular sodium (145mM Na^+/ 4mM K^+) solutions are used. Currents elicited by a family of 0.5 second voltage steps from a -30mV holding potential, from -100mV to 60mV, in 10mV increments. Currents normalized to cell capacitance (pF). Negative and positive currents indicate cation flow into or out of the cell, respectively. **(A)** Plots from HEK293 cells acutely transfected with *peel-1*::eGFP or *pmpl-1*::mCherry. **(B)** Plots from Control cell line expressing constitutive eGFP and tetracycline-inducible *pmpl-1*::mCherry. **(C)** Plots from Experimental cell line expressing constitutive *peel-1*::eGFP and tetracycline-inducible *pmpl-*

1::mCherry. Control and Experimental cell lines are shown without tetracycline or with tetracycline at the indicated time after addition of tetracycline. All plots shown as mean with SEM. **(D)** Western blot for PMPL-1::mCherry in Control and Experimental cell lines without tetracycline (-tet, left) and 18 hours after addition of tetracycline (+tet, right). Leaky expression of PMPL-1 is seen in both cell lines in the absence of tetracycline. Less background PMPL-1 expression is seen in Experimental cells than in Control cells, likely because of selection against higher background PMPL-1 expression when in combination with PEEL-1 but not eGFP. GAPDH loading control shown. **(E)** Permeability of indicated ions was assayed in Experimental cells without tetracycline. Test ionic solutions substituted previous bath solution (145mM Na^+/ 4mM mM K^+) with 140mM pure cations (external) or 20mM anions (internal), except Ca^{2+} (20mM external, 1mM internal Ca^{2+} with 2.5mM EGTA). All plots show mean with SEM. Statistical tests compare all results to NMDG (treated as control) in one-way ANOVA with Dunnett's multiple comparisons test (*, $p < 0.05$; ***, $p < 0.001$). **(F)** Schematic of planar lipid bilayer experiment. Liposomes containing purified PEEL-1 or PMPL-1 are added to the *cis* well to deliver proteins to the lipid bilayer. **(G)** Conductance traces of one experiment (left) and a histogram of the trace (right, 2pS bin width, normalized based on probability density) at an applied voltage of +180mV. SDS-PAGE gels of purified proteins are shown in fig. 2.S15 and more example conductance traces and controls shown in fig. 2.S16.

2.7 SUPPLEMENTARY MATERIAL

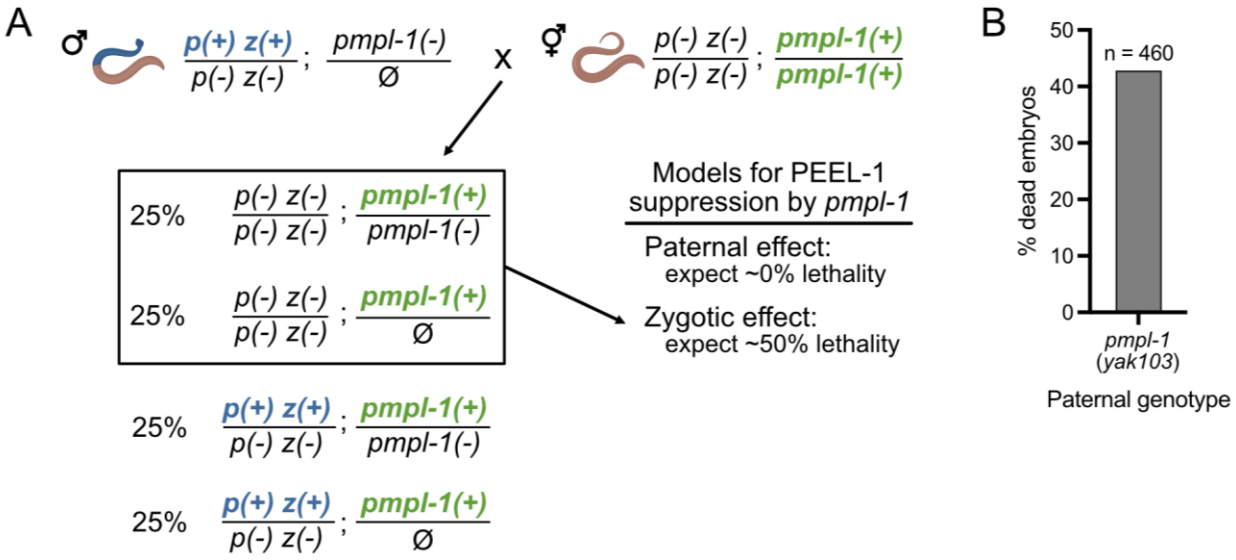

Fig. 2.S1. *pmpl-1(yak103)* does not act through paternal-effect

(A) Genetic cross to test for paternal versus zygotic effect of sperm-delivered PEEL-1 suppression by *pmpl-1(yak103)* deletion mutant (denoted *pmpl-1(-)*). Males heterozygous for the selfish element and carrying *pmpl-1(-)* are mated to *p(-) z(-); pmpl-1(+)* hermaphrodites. Suppression via paternal effect would result in ~0% embryonic lethality while suppression by a zygotic effect would result in ~50% lethality. **(B)** The percent of dead embryos seen from the cross shown in panel (A). Results are consistent with a model of *pmpl-1*(*yak103*) suppression of PEEL-1 through zygotic effect.

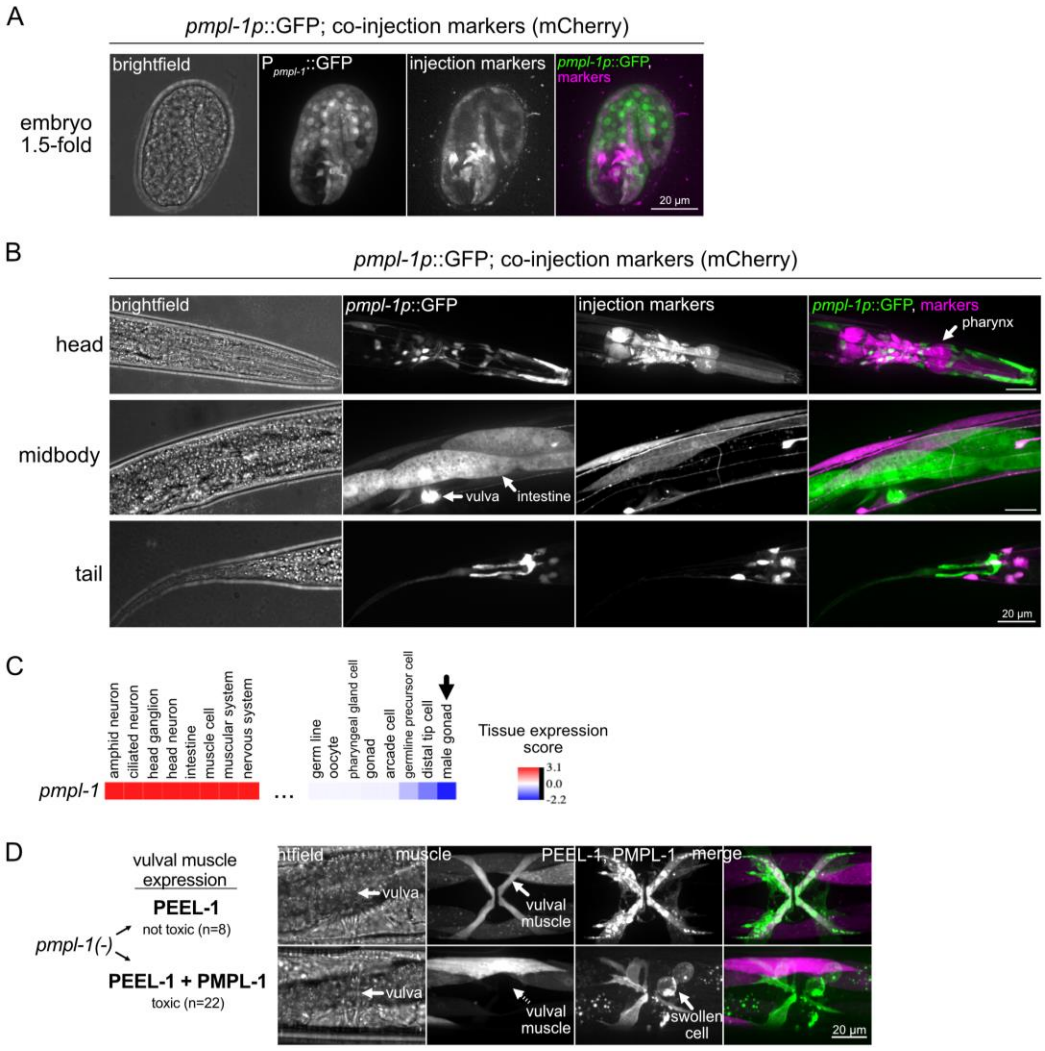

Fig. 2.S2. *pmpl-1* expression pattern in C. elegans.

(A) Maximum intensity projection of a *C. elegans*, 1.5-fold embryo with GFP driven by the *pmpl-1* promoter and co-injection markers *myo-2p*::mCherry, *rab-3p*::mCherry, and *myo-3p*::mCherry. Toxicity from sperm-delivered PEEL-1 occurs after this embryonic stage. **(B)** Maximum intensity projections of hermaphrodite adult head (top), midbody (middle), and tail (bottom) of the same strain shown in panel (A). **(C)** Heatmap of *pmpl-1* of tissue expression scores from RNA-seq dataset of Day 1 adult worms from Kaletsky et al., 2018. Tissues with the

highest *pmpl-1* expression (red) and lowest expression (blue) are shown. Only the 8 most highly expressed tissues (left) and the 8 most lowly expressed tissues (right) are shown. Expression of *pmpl-1* is lowest in the male gonad (arrow). **(D)** *pmpl-1(yak103)* worms with vulval muscle cell expression of *peel-1*::GFP alone (top) or with *pmpl*-1::GFP (bottom). Number of scored worms are indicated. Green channel brightness is increased in the bottom panel to show fluorescence in the vulval muscle, since toxicity in this cell likely caused decreased levels of fluorescent-tagged proteins.The vulval muscle appears swollen in the green channel when co-expressing *peel-1* and *pmpl-1*. This is the same worm as shown in Figure 2.1E.

Fig. 2.S3. PMPL-1 is conserved in nematodes.

Alignment of PMPL-1 amino acid sequences (right) between representative *Caenorhabditis* species, *Pristionchus pacificus*, and *Toxocara canis* shown in a species phylogeny (left). The *pmpl-1* (*yak52*) allele is mutated at conserved residue A47 (*).

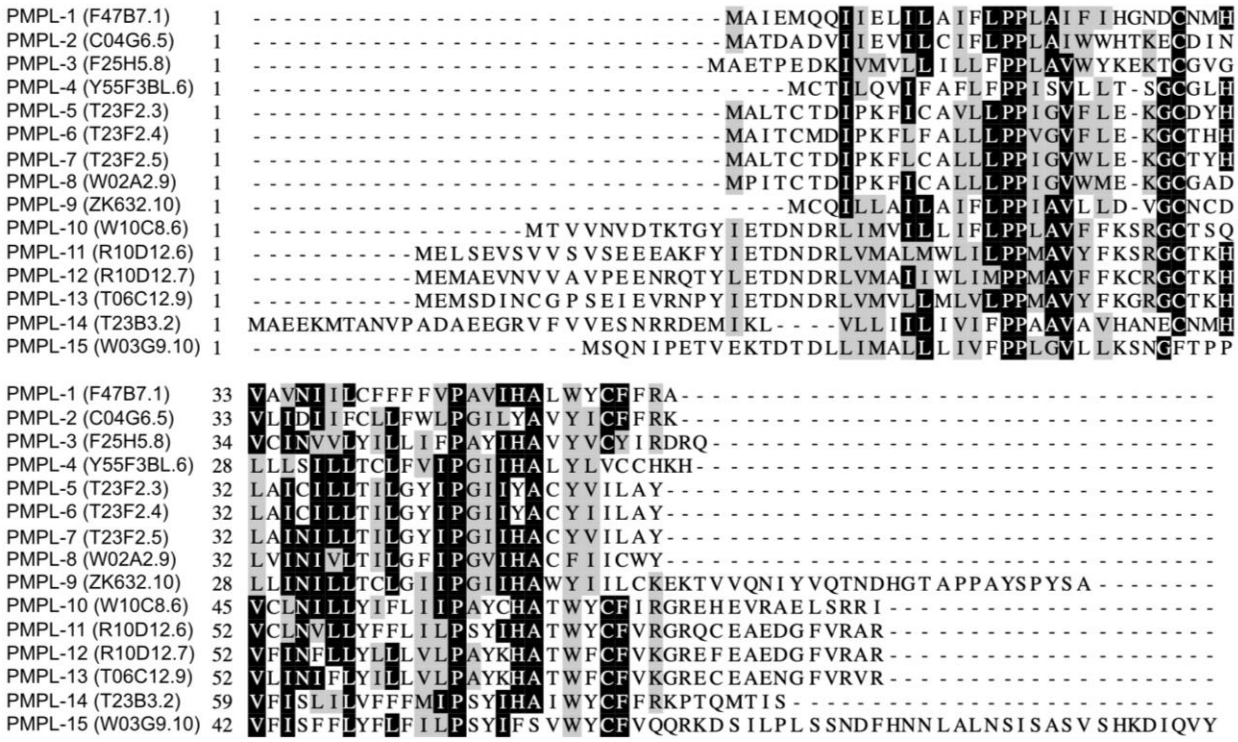

Fig. 2.S4. 15 PMP3-like proteins in C. elegans.

Alignment of all 15, PMP3-like proteins in C. elegans identified by BLAST.

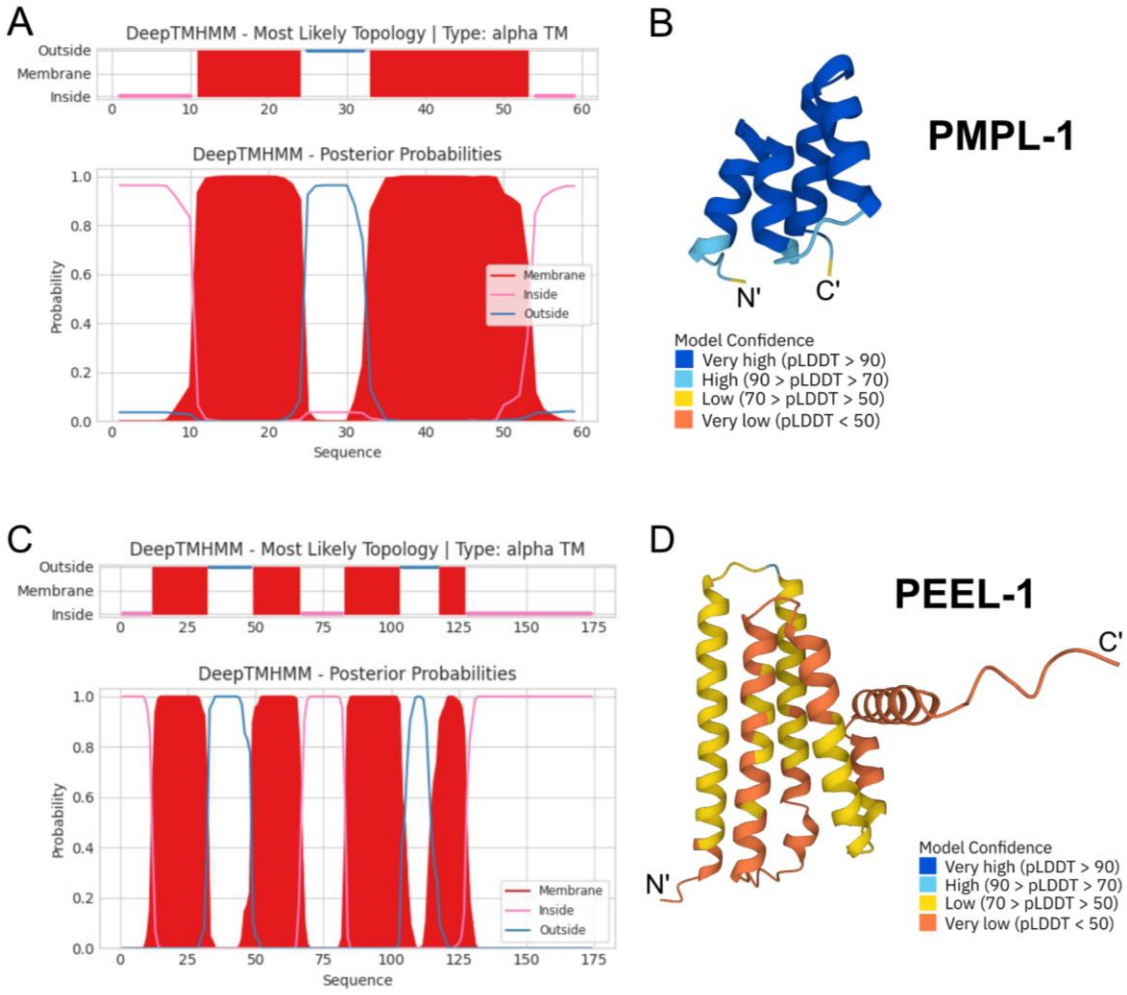

Fig. 2.S5. Structural predictions of PEEL-1 and PMPL-1.

DeepTMHMM predictions of **(A)** PMPL-1 and **(C)** PEEL-1. PMPL-1 is predicted to be a two-pass transmembrane protein. PEEL-1 is predicted to be a four-pass transmembrane protein. Both proteins are predicted to have their N- and C-termini facing the cytosol. Structural predictions from AlphaFold2 of **(B)** PMPL-1 and **(D)** PEEL-1. AlphaFold2 predicts a very high-confidence PMPL-1 structure that suggests it is a monotopic protein (passing through one leaflet of a lipid bilayer). The PEEL-1 structure is of low and very-low confidence, likely because there are no known homologs of this protein.

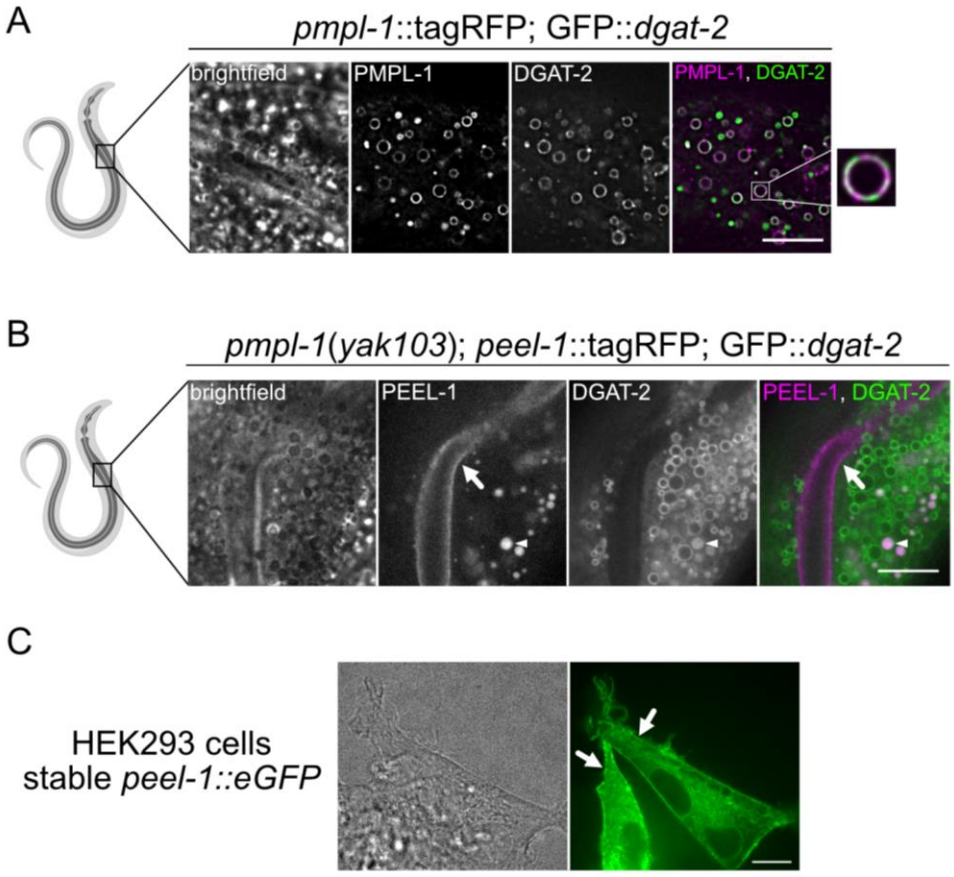

Fig. 2.S6. PEEL-1 and PMPL-1 localization.

(A-B) Intestinal cells of an adult *C. elegans* with the indicated constructs. **(A)** Wild-type worms expressing *pmpl-1*::tagRFP and GFP::*dgat-2*. DGAT-2 localizes to lipid droplet membranes, and PMPL-1::tagRFP co-localizes to these organelles. Inset shows one lipid droplet. **(B)** *pmpl-1*(*yak103*) expressing GFP::*dgat-2* and *peel-1*::tagRFP. PEEL-1 signal (arrow) appears on plasma membrane lining the intestinal lumen and does not co-localize with lipid droplets. Autofluorescence from gut granules appears as filled-in circles in both channels (arrowhead). **(C)** HEK293 cells stably expressing *peel-1*::eGFP. PEEL-1::eGFP localizes to the ER and plasma membrane (arrows). Scale bar = 10µm.

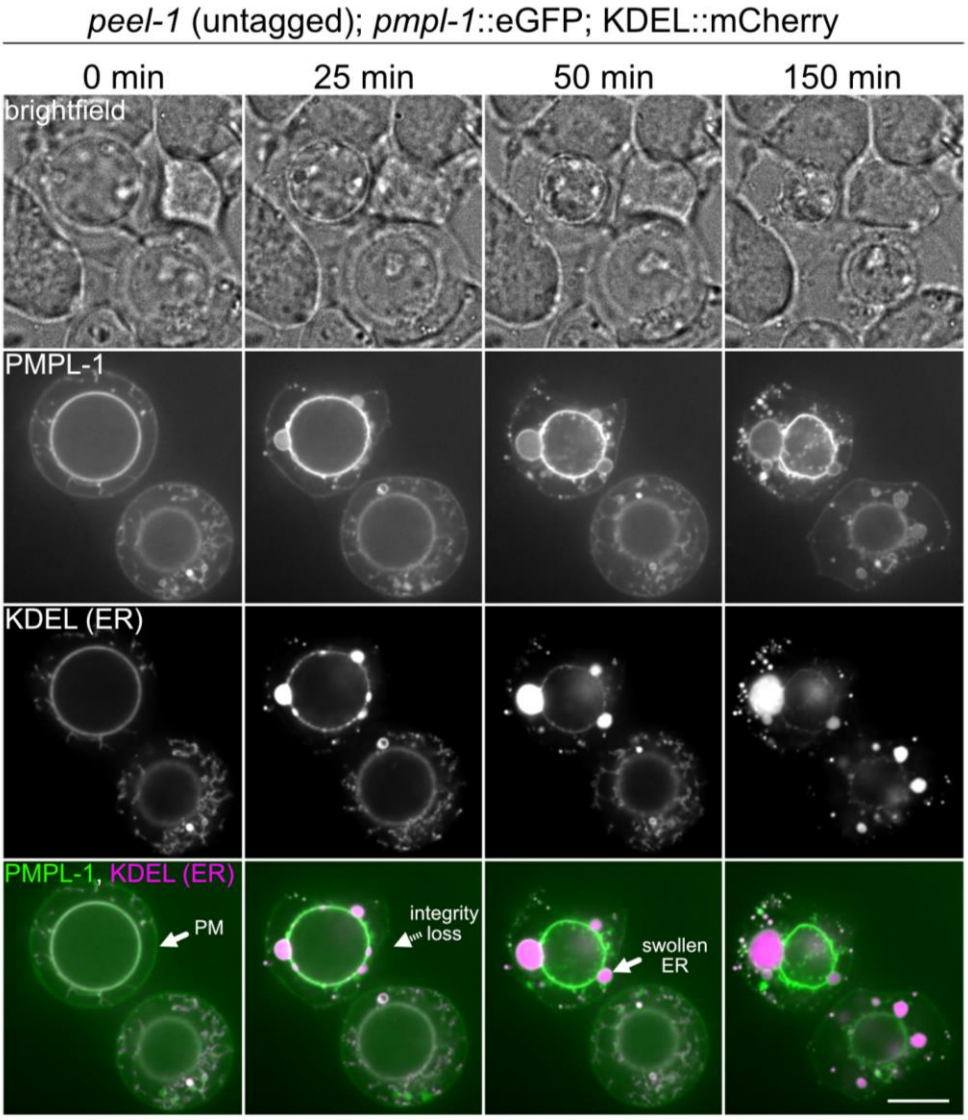

Fig. 2.S7. Toxicity causes ER swelling, ER fragmentation, and cell lysis.

Live-cell imaging time course of two HEK293T cells transfected with constructs coding for PEEL-1 (untagged), PMPL-1::eGFP, and mCherry::KDEL (ER marker). Imaging began at 20 hours post-transfection (t=0min).The top cell experiences a loss of plasma membrane (PM) integrity at 25 min, followed by ER swelling. The lower cell loses PM integrity at 150 min. Scale bar = 10μm.

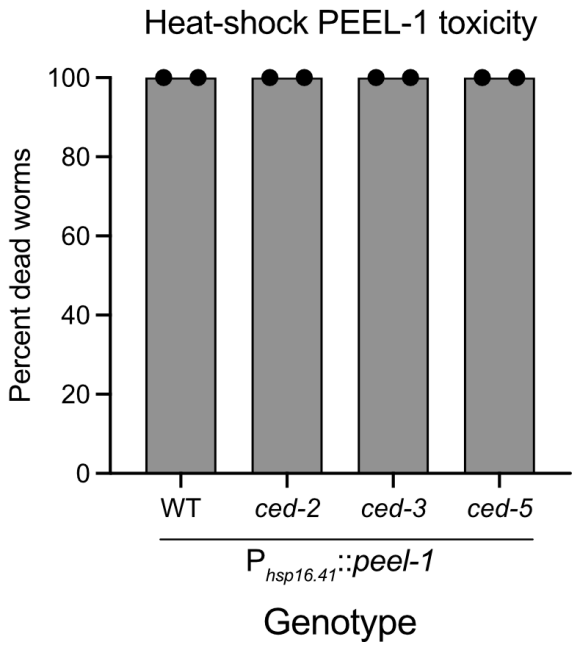

Fig. 2.S8. Ectopic PEEL-1 toxicity is non-apoptotic.

Percent dead worms after heat-shock induced expression of PEEL-1. Worms deficient in apoptosis (*ced-3*) and cell engulfment (*ced-2* and *ced-5*) were tested. Two independent experiments were done, with n=50 worms for each data point.

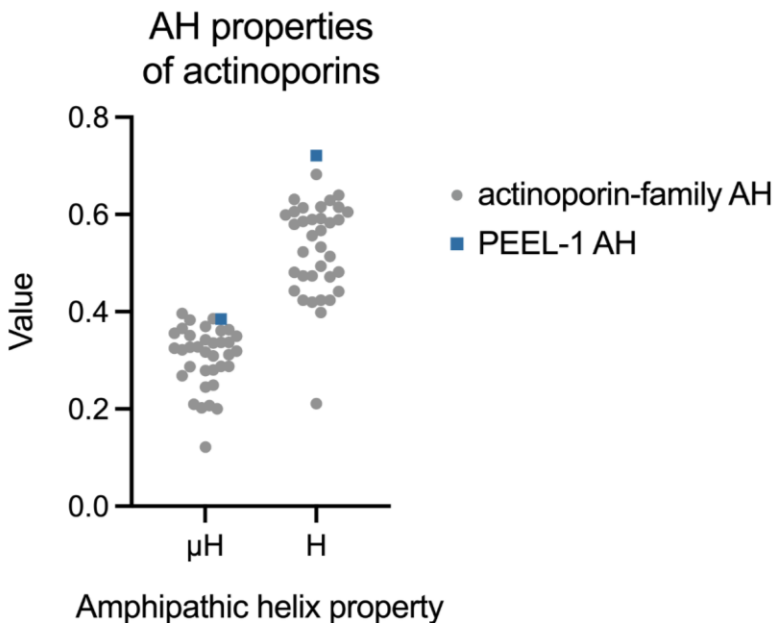

Fig. 2.S9. Amphipathic helix properties of Actinoporin toxins.

Hydrophobic moment (μH) and hydrophobicity (H) of the amphipathic helix of 35 Actinoporin toxin proteins (data acquired from Macrander and Marymegan et al., 2016) (gray circles) and the predicted PEEL-1 amphipathic helix (blue square).

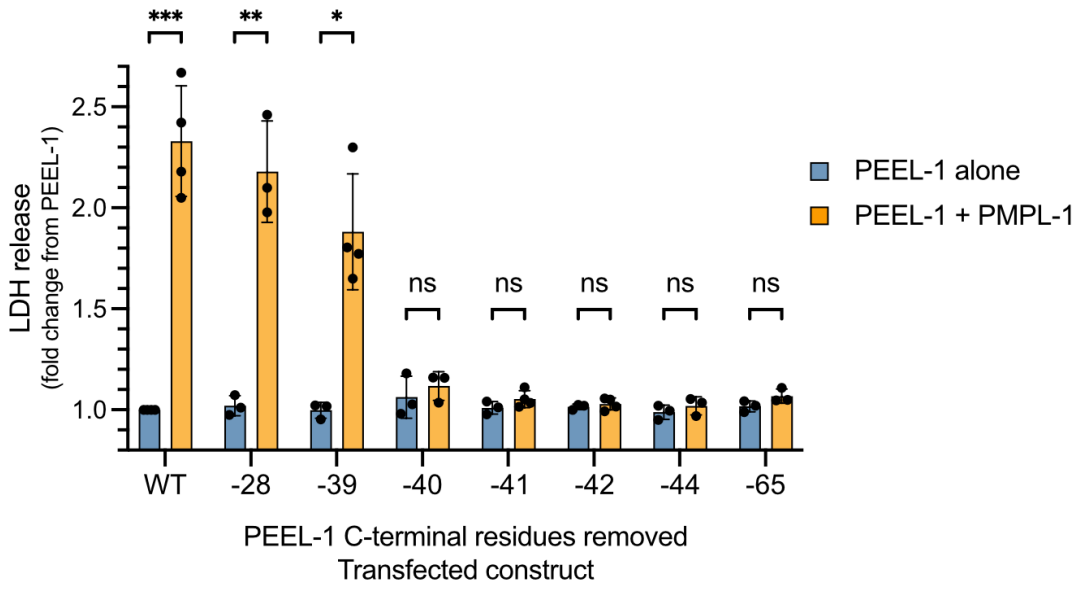

Fig. 2.S10. Toxicity of PEEL-1 C-terminal truncations.

Cytotoxicity of PEEL-1 C-terminal truncations assayed alone (blue bars) or with PMPL-1 (yellow bars) in HEK293T cell transfections. The number of amino acids removed from the C-terminus is indicated (ex. -28 means 28 amino acids were removed). Data from PEEL-1 WT, -28, -39, and -65 are from Figure 4C. Toxicity is lost upon removal of the -40 residue (Ala135). Statistical tests done using multiple unpaired t-tests with Holm-Šídák test, comparing each PEEL-1 alone to PEEL-1 and PMPL-1 (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

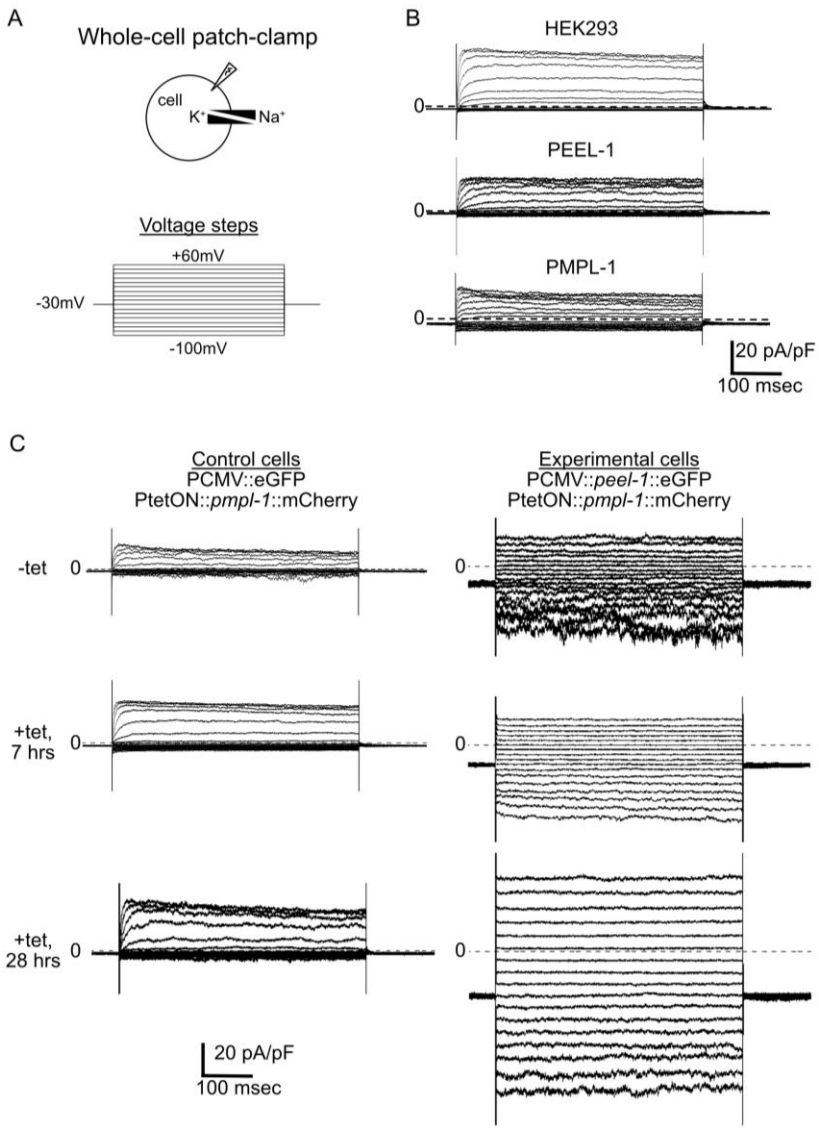

Fig. 2.S11. Raw traces of HEK293 electrophysiology experiments.

(A) Schematic of electrophysiology experiment. High intracellular potassium (140mM K^+/ 8.6mM Na^+) and high extracellular sodium (145mM Na^+/ 4mM K^+) solutions are used. Currents elicited by a family of 0.5 second voltage steps from a -30mV holding potential, from -100mV to 60mV, in 10mV increments. Current traces are normalized to capacitance (pF). **(B)** Representative traces of untransfected HEK293 cells (top) and HEK293 cells transfected with *peel-1*::eGFP (middle) or *pmpl-1*::mCherry (bottom). Scale bar shown (bottom-right). **(C)** Representative traces of tetracycline-inducible cells lines. Control cells (left) have stable

expression of eGFP and inducible expression of *pmpl-1*::mCherry. Experimental cells (right) have stable expression of *peel-1*::eGFP and inducible expression of *pmpl-1*::mCherry. Recordings of cell lines without induction (top), 7 hours after tetracycline addition (middle), and 28 hours after tetracycline addition (bottom) are shown.

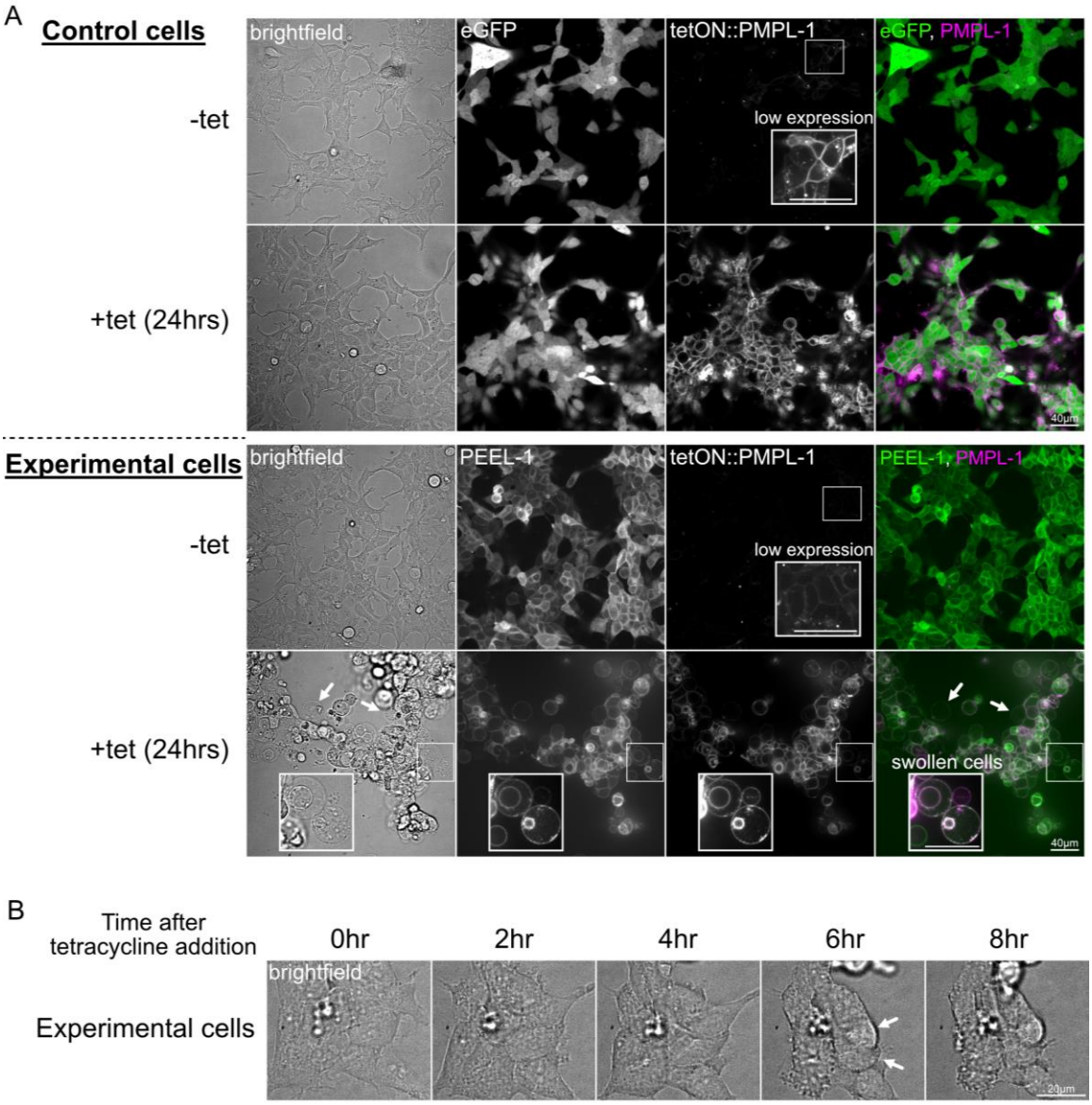

Fig. 2.S12. Tetracycline-induced toxicity in stable cell lines.

(A) Live-cell images of Control cells (CMV-driven eGFP; tetON::*pmpl-1*::mCherry) and Experimental cells (CMV-driven *peel-1*::eGFP; tetONp::*pmpl-1*::mCherry). Cell lines are shown without tetracycline (-tet) and 24 hours after tetracycline addition (+tet (24hrs)). Insets in -tet conditions show leaky expression of PMPL-1 in both cell lines (LUT adjusted within inset). Images show successful tetracycline-inducible expression of PMPL-1::mCherry and efficient tetracycline-induced killing in experimental cells but not control cells. Arrows and inset in

experimental cells +tet (24hrs) show examples of swollen cells. Exposure time in the green channel is different between cell lines. Scale bar = 40μm. **(B)** Time course of toxicity after addition of tetracycline to experimental cells. Noticeable cell swelling is seen after 6 hours (arrows). Some acute swelling may also be visible at 4 hours after addition of tetracycline. Scale bar = 20μm.

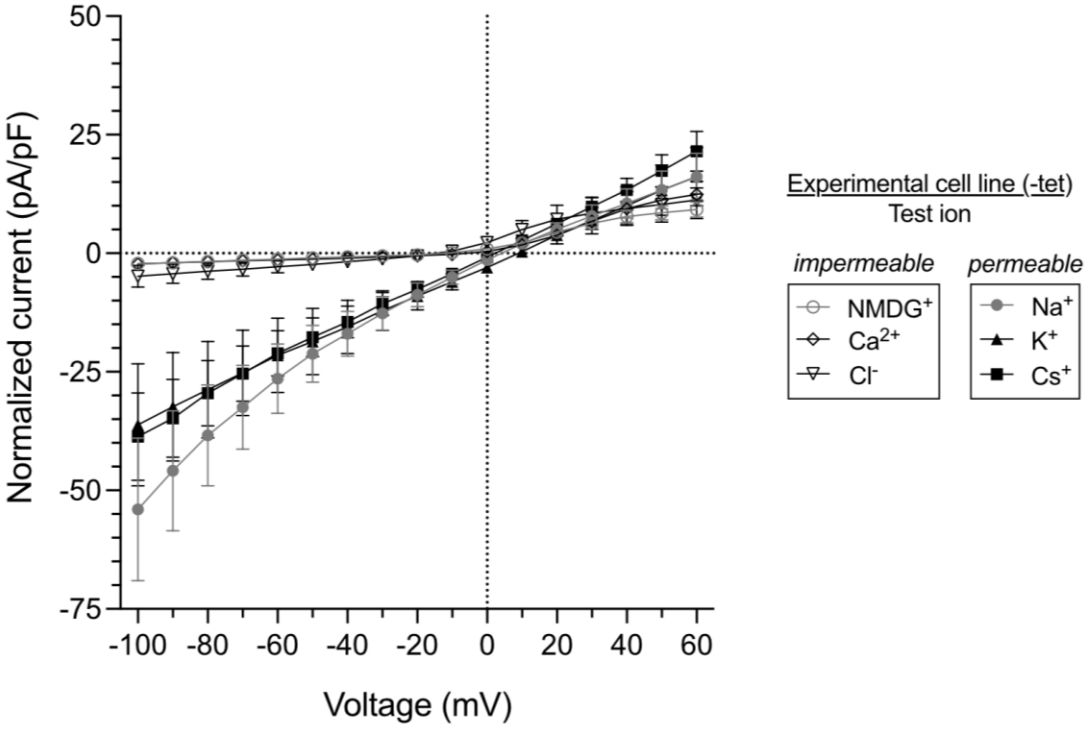

Fig. 2.S13. Ion permeabilities in uninduced experimental cells.

Current-voltage plots of experimental cells without tetracycline in different ionic conditions to test permeabilities of the indicated ions. Permeable ions have greater inward currents (Na^+, K^+, Cs^+; closed symbols) compared to impermeable ions ($NMDG^+$, Ca^{2+}, Cl^-; open symbols) at negative voltages. Mean with SEM is shown. These data are summarized in Fig. 2.5E.

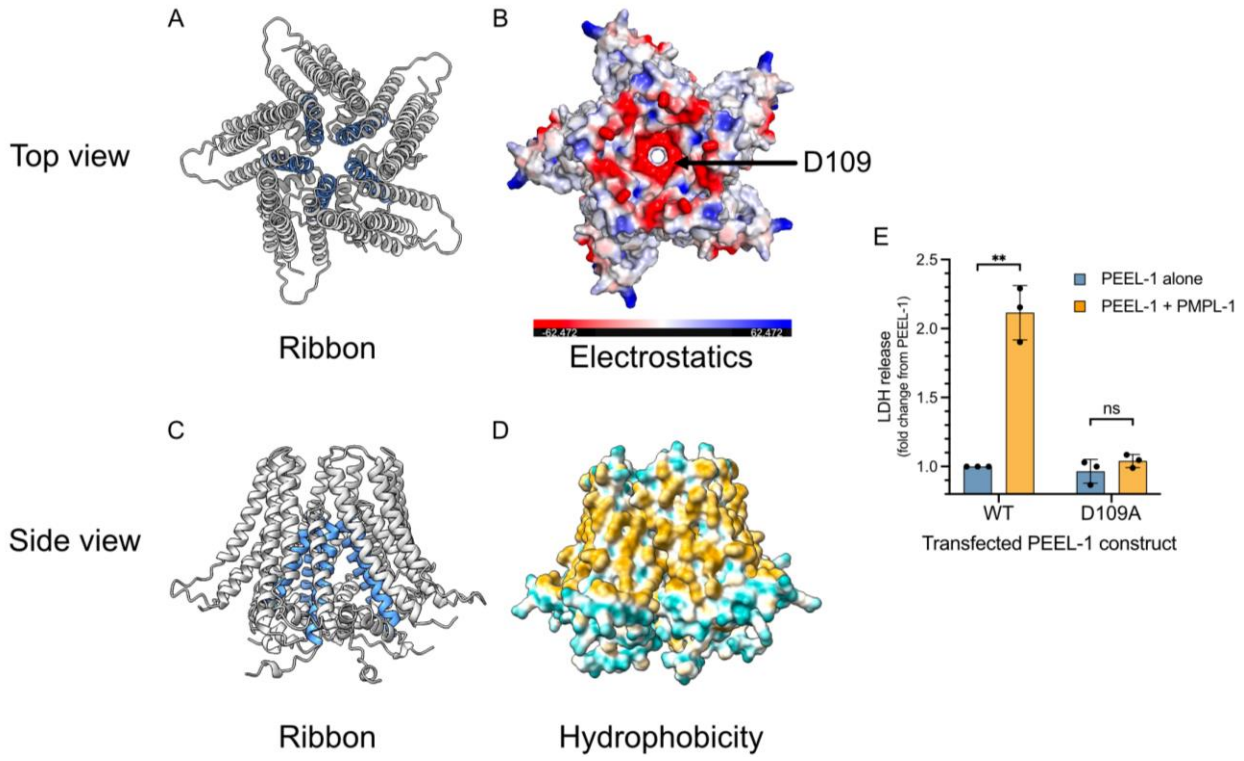

Fig. 2.S14. Predicted PEEL-1 pentameric structure.

AlphaFold2 prediction of the PEEL-1 pentameric structure is shown. Two angles are shown: **(A-B)** top view, showing the predicted extracellular face of the complex, and **(C-D)** side view, in the plane of the lipid bilayer. **(A and C)** Ribbon diagram with the amphipathic helix colored in blue creating the lining of a pore-like region. **(B)** Surface representation of electrostatic predictions (red = negative charge, blue = positive charge). An uninterrupted hole can be seen through the structure, with a ring of negative charge from five D109 residues. **(D)** Surface representation of hydrophobicity (yellow = hydrophobic, cyan = hydrophilic). **(E)** Cytotoxicity of mutants which eliminate the predicted ring of negative charge at the top of the complex via a D109A mutation. Statistics performed using multiple unpaired t-tests with Holm-Šídák test. All tested comparisons are shown.

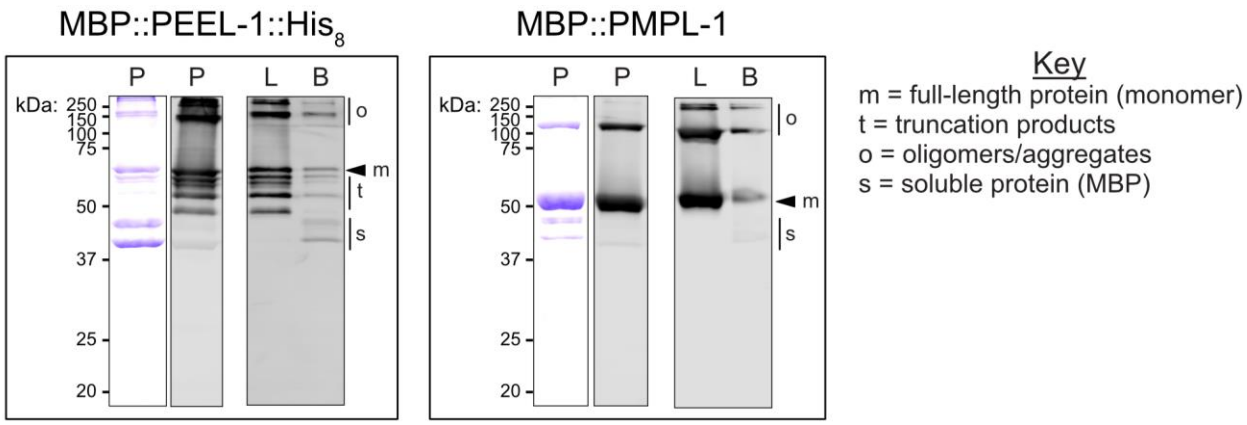

Fig. 2.S15. PEEL-1 and PMPL-1 purification and liposomes.

SDS-PAGE gels of indicated constructs after purification (P; Commassie Blue stain and anti-MBP western blot) and after incorporation into liposomes (L; anti-MBP western blots). Liposomes were collected after flotation through a density gradient before use in synthetic bilayer experiments. The top fraction contains liposomes (L) and the bottom fraction (B) contains unincorporated protein. Monomers (m), oligomers or aggregates (o), and truncation products of PEEL-1 (t) are indicated. The bottom bands seen in both MBP-tagged PEEL-1 and PMPL-1 purifications are soluble proteins (s) that did not get incorporated into liposomes, likely endogenous MBP (43kDa) or truncated MBP-tagged protein.

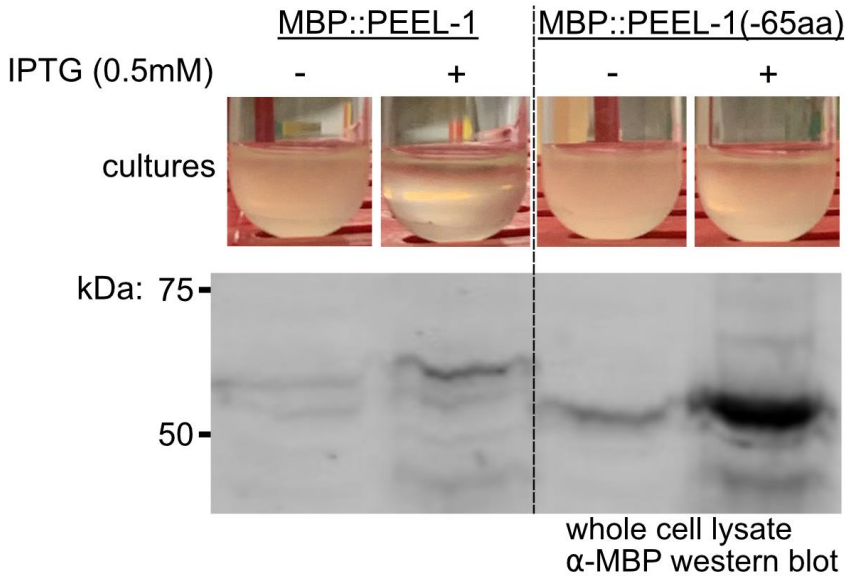

Fig. 2.S16. PEEL-1 is toxic to bacteria and requires the amphipathic helix.

E. coli C41(DE3) cells with constructs encoding IPTG-inducible expression of either MBP::PEEL-1 (left) or MBP::PEEL-1(-65aa) (right). Images of cultures are taken after shaking at 18°C overnight, with or without 0.5mM IPTG. Cultures appear lysed when full-length PEEL-1 is expressed but not PEEL-1(-65aa) which lacks the PEEL-1 AH. Western blot (anti-MBP) of corresponding whole-cell lysates are shown (bottom), confirming higher expression of MBP::PEEL-1(-65aa). This experiment was repeated four times and yielded similar results.

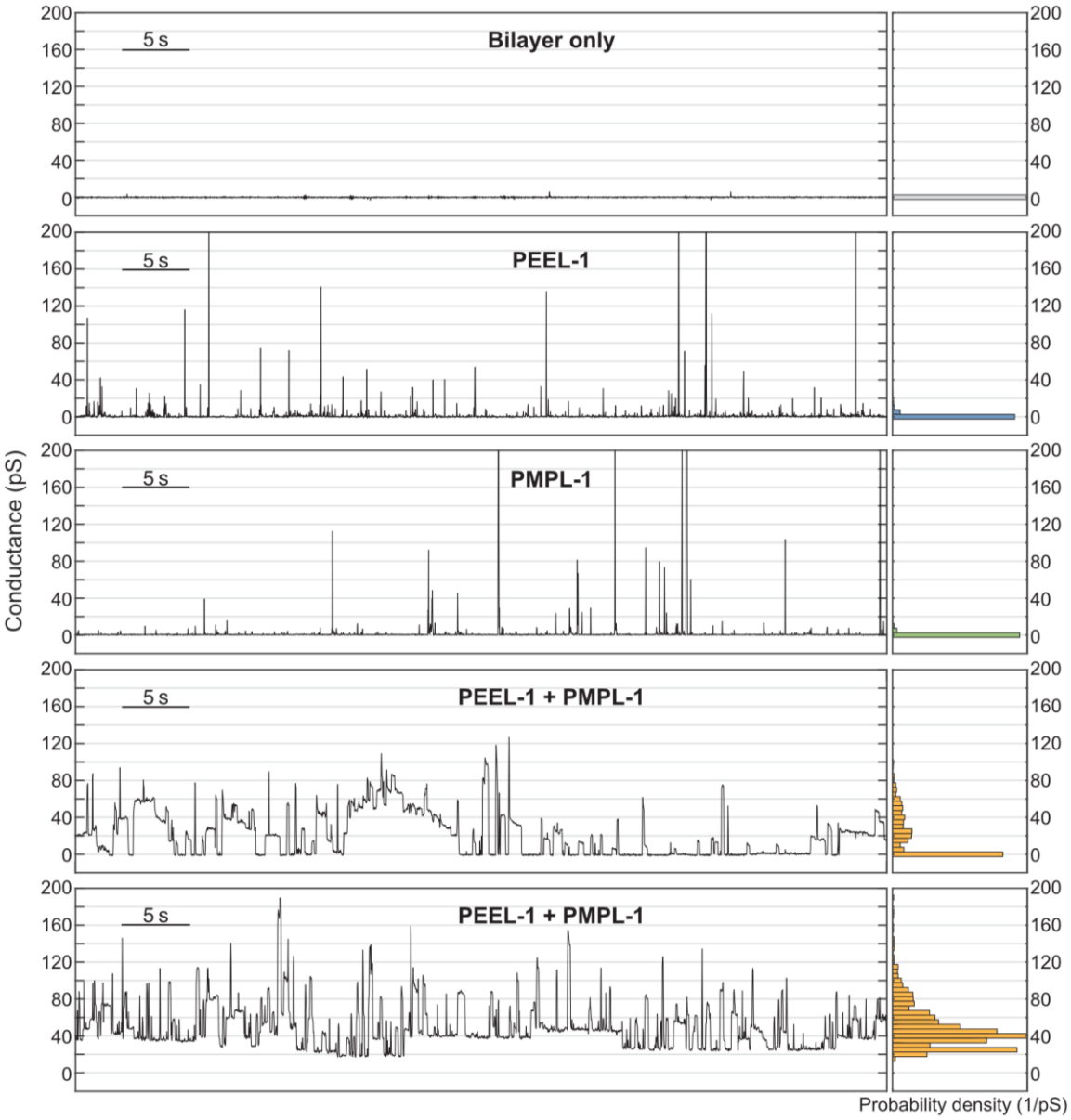

Fig. 2.S17. Purified PEEL-1 and PMPL-1 conduct ions through planar lipid bilayers.

Conductance traces through artificial planar lipid bilayers are shown. (top to bottom) Bilayer alone without addition of liposomes, bilayer with PEEL-1 liposomes added (transient spikes indicate successful liposome fusions), bilayer with PMPL-1 liposomes added, and two independent experiments of bilayers with PEEL-1 liposome and PMPL-1 liposomes added. An all-point histogram is shown (right, 2pS bin width, normalized based on probability density).

Channel activity after addition of PEEL-1 and PMPL-1 was observed in 8 independent experiments. A voltage of -180mV was applied to the bilayer for liposome fusions in all experiments. After observing channel activity, a voltage of +180mV was applied since channel activity was more stable at positive voltages. Bottom two panels show traces at +180mV. Scale bar = 5 seconds.

Table 2.S1. *C. elegans* strains used in this study.

Strain ID	Strain genotype	Source
N2	N2	CGC
XZ1372	yakTi4[hsp16.41p::eGFP::his-44 , NeoR] I ; oxSi507[hsp-16.41p::peel-1, Cb-unc-119] II ; oxSi280[hsp16.41p::peel-1, Cb-unc-119] IV	Crawford et al., 2023
XZ1047	oxSi507[hsp-16.41p::peel-1, Cb-unc-119] II ; unc-119(ed9) III ; oxSi280[hsp16.41p::peel-1, Cb-unc-119] IV ; him-5(e1490) V	Crawford et al., 2023
XZ103	oxSi507[hsp-16.41p::peel-1, Cb-unc-119] II ; oxSi280[hsp16.41p::peel-1, Cb-unc-119] IV ; pmpl-1(yak103) X	this study
XZ2283	oxSi507[Phsp-16.41::peel-1, Cb-unc-119] II ; oxSi280[Phsp16.41::peel-1, Cb-unc-119] IV ; F47B7.1(yak52) X	this study
AFS216	zeel-1(tm3419) I peel-1(cle6) I	Aaron Severson
XZ1177	oxSi507[hsp-16.41p::peel-1, Cb-unc-119] II ; unc-119(ed9) III ; oxSi280[hsp16.41p::peel-1, Cb-unc-119] IV ; him-5(e1490) V ; pmpl-1(yak52) X	this study
XZ1307	oxSi507[hsp-16.41p::peel-1, Cb-unc-119] II ; oxSi280[hsp16.41p::peel-1, Cb-unc-119] IV ; him-5(e1490) V ; pmpl-1(yak103) X	this study
EG1000	dpy-5(e61) I ; rol-6(e187) II ; lon-1(e1820) III	Erik M. Jorgensen
EG1020	bli-6(sc16) IV ; dpy-11(e224) V ; lon-2(e678) X	Erik M. Jorgensen
EG8040	oxTi302[Peft-3::mCherry cb-unc-119(+)] I ; oxTi75[Peft-3::GFP::H2B::tbb-2utr unc-18(+)] II ; oxTi411[Peft-3::TdTomato::H2B::unc-54 cb-unc-119(+)] III ; him-8(e1489) IV	Jorgensen lab
EG8041	oxTi76[Peft-3::GFP::H2B::tbb-2utr unc-18(+)] IV ; oxTi405[Peft-3::TdTomato::H2B::unc-54 cb-unc-119(+)] V him-5(e1490) V ; oxTi421[Peft-3::mCherry cb-unc-119(+)] X	Jorgensen lab
XZ2194	pmpl-1 (yak103) X	this study
XZ2103	oxSi507[hsp-16.41p::peel-1, Cb-unc-119] II ; ced-3(n717) IV ; him-5(e1490) V	this study
XZ2102	oxSi507[hsp-16.41p::peel-1, Cb-unc-119] II ; ced-5(n1812) IV	this study
XZ2096	oxSi507[hsp-16.41p::peel-1, Cb-unc-119] II ced-2(n1994) IV	this study
XZ2254	yakEx195[pmpl-1p::GFP; myo-2p::mcherry; myo-3p::mcherry; rab-3p::mCherry]	this study
XZ2276	pmpl-1(yak103) X ; yakEx203[exp-3p::peel-1::GFP, myo3p::mCherry]	this study
XZ2633	pmpl-1(yak103) X ; yakEx275[exp-3p::peel-1::GFP, exp-3p::pmpl-1::GFP,	this study
XZ2551	yakEx264[hsp16.41p::peel-1(-28aa), cc::GFP]	this study
XZ2634	yakEx276[hsp16.41p::peel-1(-39aa), cc::GFP]	this study
XZ2548	yakEx263[hsp16.41p::peel-1(-65aa), cc::GFP]	this study
XZ2454	pmpl-1(yak103) X ; yakEx275[exp-3p::peel-1::GFP, exp-3p::pmpl-1::GFP, myo3p::mCherry]	this study
XZ2452	hjSi56[Pvha-6::3xFLAG::TEV::GFP::dgat-2::let-858 3' UTR] IV ; yakEx242[vha-6p::pmpl-1::tagRFP, myo-2p::mCherry]	this study

Table 2.S2. Constructs used in this study.

Construct ID	Description	resistance	sequenced?	notes
pLC4	exp-3p::pmpl-1::tagRFP::tbb-2 3'UTR	carb		
pLC6	exp-3p::peel-1::GFP::tbb-2 3'UTR	carb		
pLC26	MBP::TEV::peel-1	carb	sequenced	
pLC28	MBP::TEV::pmpl-1	carb	sequenced	
pLC31	exp-3p::pmpl-1::GFP tbb-2 3'UTR	carb		
pLC37	PMP3(S. cerevisiae)::mCherry_N1	kan	sequenced	
pLC38	mCherry::zeel-1_N1	kan		
pLC54	pmpl-1::eGFP_N1	kan		
pLC65	MBP::TEV::peel-1	carb+chlor	sequenced	
pLC67	MBP::TEV::pmpl-1	carb+chlor	sequenced	
pLC79	tetON::pmpl-1::mCherry_pFTSH	carb	sequenced	
pLC84	exp-3p::pmpl-1::tagRFP::tbb-2 3'UTR	carb		
pLC103	vha-6p::pmpl-1::tagRFP::tb-2 3'UTR	carb		
pLC113	vha-6p::peel-1::tagRFP::tbb-2 3'UTR	carb		
pLC122	pmpl-2::mCherry_N1	kan	sequenced	
pLC123	pmpl-1(A47T)::mCherry_N1	kan	sequenced	
pLC124	peel-1(S124F)::eGFP_N1	kan	sequenced	
pLC172	hsp16.41p::peel-1(-28aa)::let-858 3'UTR	carb	sequenced	
pLC173	hsp16.41p::peel-1(-65aa)::let-858 3'UTR	carb	sequenced	
pLC174	peel-1(-28aa)_N1	kan	sequenced	
pLC175	peel-1(-65aa)_N1	kan	sequenced	
pLC226	peel-1(-39aa)_N1	kan	sequenced	
pLC227	MBP::TEV::peel-1(-65aa)	carb	sequenced	
pLC294	MBP::TEV::peel-1 (-65aa)	carb/chlor	sequenced	
pLC297	peel-1(-42aa)_N1	kan	sequenced	
pLC298	peel-1(-44aa)_N1	kan	sequenced	
pLC304	peel-1(-41aa)_N1	kan	sequenced	
pLC305	peel-1(-40aa)_N1	kan	sequenced	
pLC345	peel-1::eGFP::ER-ret(GBR1 C-tail)_N1	kan	sequenced	
pLC363	peel-1(S124V)::eGFP_N1	kan	sequenced	
pLC365	pmpl-1::mCherry::ER-ret(GBR1 C-tail)_N1	kan	sequenced	
pLC370	MBP::TEV::peel-1::8X His	carb	sequenced	
pLC376	MBP::TEV::peel-1::8X His	carb/chlor	sequenced	
pLC385	peel-1(D109A)::eGFP_N1	kan	sequenced	
pLC395	peel-1(L115Q)::eGFP_N1	kan	sequenced	
pLC396	peel-1(L118Q)::eGFP_N1	kan	sequenced	
pLC397	peel-1(L122Q)::eGFP_N1	kan	sequenced	
pLC398	peel-1(L126Q)::eGFP_N1	kan	sequenced	
pLC438	peel-1(L118Q,S124V)::eGFP_N1	kan	sequenced	
pLC439	peel-1(L118Q,L126Q)::eGFP_N1	kan	sequenced	
pLC440	peel-1(S124V,L126Q)::eGFP_N1	kan	sequenced	
pLC475	hsp16.41p::peel-1(-39aa)::let-858 3'UTR	carb	sequenced	
mCherry-KDEL	mCherry-KDEL	kan		gift from Suzanne Hoppins
pGP9	peel-1::eGFP_N1	kan	sequenced	
pGP10	pmpl-1::mCherry_N1	kan	sequenced	
pPD97/98	cc::GFP (unc-122p::GFP)	carb		gift from Piali Sengupta
pCFJ90	Pmyo-2::mCherry::unc-54 3'UTR	carb		
pCFJ104	Pmyo-3::mCherry::unc-54 3'UTR	carb		
pGH8	Prab-3::mCherry::unc-54 3'UTR	carb		
pBS_SK	pBluescript	carb		
pCFJ150	destination vector (4-1-2-3)	carb		
eGFP_N1	eGFP_N1	kan		gift from Suzanne Hoppins
mCherry_N1	mCherry_N1	kan		gift from Suzanne Hoppins

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Chapter 3. RECURRENT BUT SHORT-LIVED DUPLICATIONS OF CENTROMERIC PROTEINS IN HOLOCENTRIC *CAENORHABDITIS* SPECIES

This chapter is closely adapted from Caro *et al.,* 2022. The work in this chapter was done in close collaboration with Pravrutha Raman (Fred Hutch Cancer Center), Florian Steiner (University of Geneva), and Harmit S. Malik (Fred Hutch Cancer Center).

3.1 INTRODUCTION

The faithful inheritance of genetic material is indispensable for all life. In most eukaryotes, faithful inheritance of chromosomes relies on the centromeric histone H3 variant (*CenH3*) to attach chromosomes to microtubules. CenH3 acts both as a structural component of the multi-subunit complex that links chromosomes to microtubules for segregation and as the epigenetic mark that defines and maintains the centromeric location(s) on chromosomes (Allshire and Karpen 2008; De Wulf and Earnshaw 2008; Fukagawa and Earnshaw 2014; McKinley and Cheeseman 2016; Ali-Ahmad and Sekulić 2020; Mellone and Fachinetti 2021). CenH3 is critical for chromosome segregation during mitosis and meiosis. Mutations or misregulation of CenH3 have severe consequences for fertility and viability in many species (Stoler, et al. 1995; Buchwitz, et al. 1999; Howman, et al. 2000; Blower and Karpen 2001). CenH3 would therefore be expected to be conserved across eukaryotes and expected to evolve under strong evolutionary constraints to maintain functionality.

Despite this expectation for strong conservation, *CenH3* genes have rapidly evolved in animal and plant species (Malik and Henikoff 2001; Talbert, et al. 2004; Schueler, et al. 2010).

This rapid evolution is hypothesized to result from a unique genetic conflict that stems from asymmetric female meiosis in animals and plants, in which only one of four meiotic products gets selected to be included in the oocyte nucleus. As a result of this bottleneck, chromosomes compete for inclusion into the egg in a process termed 'centromere drive' (Henikoff, et al. 2001; Malik 2009; Schueler, et al. 2010; Lampson and Black 2017). This competition favors changes in centromeric DNA that result in over-recruitment of centromeric proteins (Chmátal, et al. 2014; Akera, et al. 2017; Iwata-Otsubo, et al. 2017). Conversely, genes encoding centromeric proteins evolve rapidly to suppress the 'selfish advantage' of cheating centromeres to restore parity and ameliorate the deleterious effects of centromere-drive (Finseth, et al. 2021; Kumon, et al. 2021). Thus, in many animal and plant species, CenH3 proteins evolve rapidly despite being essential for faithful chromosome segregation.

CenH3 proteins can also function differently during meiotic and mitotic segregations. Some plant *CenH3* mutants only show defects during meiosis, but not mitosis (Lermontova, et al. 2011; Ravi, et al. 2011; Schubert, et al. 2014). Conflicting evolutionary selective pressures on *CenH3* between these functions (*e.g.*, mitotic versus meiotic, conserved versus rapidly evolving) could be resolved by gene duplication, which allows the duplicate (paralog) and ancestral genes to specialize for different functions (Hittinger and Carroll 2007; Des Marais and Rausher 2008; Gallach and Betrán 2011). Indeed, *CenH3* genes have also undergone repeated gene duplications not just in plants but also in several animal species including cows, fruit flies, mosquitoes, and nematodes (Li and Huang 2008; Zedek and Bureš 2016; Kursel and Malik 2017; Ishii, et al. 2020; Kursel, et al. 2020; Despot-Slade, et al. 2021; Elisafenko, et al. 2021; Kursel, et al. 2021).

Cytological evidence in *Drosophila virilis* suggests that divergent *CenH3* paralogs can acquire separate, tissue-specific functions (Kursel, et al. 2021).

Although *CenH3* has undergone duplication and diversification in *Drosophila* and mosquito species, four orders of insects have completely lost *CenH3* (Drinnenberg, et al. 2014). *CenH3* loss appears to correlate with transitions from monocentricity, in which centromeric determinants are concentrated in one genomic region, to holocentricity, in which centromeres are dispersed along the length of their chromosomes. Thus, holocentricity may impose unique selective pressures that shape the path of *CenH3* and kinetochore evolution (Marques and Pedrosa-Harand 2016; Cortes-Silva, et al. 2020; Senaratne, et al. 2022; Wang, et al. 2022).

In contrast to holocentric insects that have lost *CenH3*, *CenH3* homologs are present in other holocentric animal and plant species (Drinnenberg, et al. 2014). Moreover, several nematode clades encode duplications and diversification of *CenH3* genes (Despot-Slade, et al. 2021). Holocentric chromosome segregation in nematodes has been best studied in *C. elegans,* which encodes two *CenH3* paralogs. The first of these to be characterized was *hcp-3*, which encodes a protein required for recruiting all other kinetochore proteins and is essential for embryonic mitotic divisions in *C. elegans* (Buchwitz, et al. 1999; Oegema, et al. 2001). However, HCP-3 appears to be dispensable for oocyte meiotic segregation (Monen, et al. 2005). A second *CenH3* paralog in *C. elegans*, CPAR-1, shares high sequence similarity to HCP-3 in the histone fold domain but is diverged in the N-terminal domain (Monen, et al. 2015). Although CPAR-1 is enriched in meiotic chromosomes, it does not appear to localize to centromeres at all, and its precise function is not well understood (Gassmann, et al. 2012; Monen, et al. 2015). An

independent *hcp-3* duplication occurred in a related species, *C. remanei* (Monen, et al. 2015), but its function is also unknown. These previous studies left unclear whether *CenH3* duplications in *C. elegans* and *C. remanei* were unusually rare or typical of *Caenorhabditis* nematodes.

Faithful chromosome segregation in *C. elegans* relies not only on CenH3 alone but also on CenH3 interaction with HCP-4 (CENP-C in mammals) and KNL-2 to form the inner kinetochore. A predicted structured region of the HCP-3 N-terminal tail interacts with KNL-2 (de Groot, et al. 2021; Prosée, et al. 2021). This interaction is necessary for the establishment of centromeres in the hermaphrodite germline, prior to the first embryonic mitosis (Prosée, et al. 2021). Identifying which HCP-3 residues are important for protein interactions has been challenging, owing to low sequence identity of CenH3 among species (de Groot, et al. 2021; Prosée, et al. 2021). Despite high sequence divergence of CenH3 N-terminal tails, *CenH3* evolution is likely constrained to maintain important protein-protein interaction interfaces (Malik, et al. 2002; Maheshwari, et al. 2015). Identifying these constraints may reveal insights into the molecular architecture of such interactions. Thus, a phylogenetic study of CenH3 and kinetochore protein evolution and duplication in *Caenorhabditis* nematodes would not only yield insights into the cadence of gene duplication and retention but also reveal functional constraints that would inform the molecular interactions that underlie the important function of chromosome segregation.

The growing collection of *Caenorhabditis* species and their genome sequences (Stevens, et al. 2019) (unpublished genomes at http://caenorhabditis.org/) provides a rich dataset for identifying both the evolutionary trajectory and constraints of their *CenH3* genes. Taking

advantage of this resource, we performed detailed phylogenomic analyses to understand the evolution of *CenH3* genes in *Caenorhabditis*. Our studies reveal that thirteen out of thirty-two analyzed *Caenorhabditis* species encode two or more *CenH3* paralogs, which were the result of at least ten independent duplication events. We confirm these paralogs are expressed in both sexes in representative species. We identify novel, conserved protein motifs within the N-terminal domains of *Caenorhabditis* CenH3 proteins that are likely important for interactions with other kinetochore proteins and for centromere biology. Although some motifs are strictly retained, others display variable instances of loss and retention between ancestral and duplicate genes, revealing clues to their sub-functionalization. In a possible case of neofunctionalization, we find an unusual *CenH3* paralog in *C. afra* that encodes a CENP-C-CenH3 fusion protein. Extending our analyses beyond *CenH3*, we find independent duplications of other inner and outer kinetochore proteins, revealing a remarkable pace of diversification of the kinetochore within *Caenorhabditis* nematodes. Our analyses thus reveal an unusual 'revolving door' of CenH3 protein duplications, with retention only over short evolutionary periods. This pattern contrasts with the strict, long-lived retention of *CenH3* paralogs seen in *Drosophila*, mosquito, plant, and even other holocentric nematode species (Maheshwari, et al. 2015; Kursel and Malik 2017; Kursel, et al. 2020; Kursel, et al. 2021, Despot-Slade, et al. 2021). We hypothesize that this pattern may result from the unusual mechanisms of centromere establishment and inheritance in holocentric *Caenorhabditis* species.

3.2 RESULTS

3.2.1 hcp-3 has duplicated at least ten independent times in Caenorhabditis

Global efforts to isolate and sequence *Caenorhabditis* species have recently resulted in several well-assembled genomes from highly diverged species (Stevens, et al. 2019) (unpublished genomes at http://caenorhabditis.org/). We used this resource for phylogenomic analyses of *CenH3* evolution. We used *C. elegans* HCP-3 as a query for tBLASTn searches against genome sequences from 32 *Caenorhabditis* species (Altschul, et al. 1990; Altschul, et al. 1997; Stevens, et al. 2019) (http://caenorhabditis.org/) to identify all *hcp-3* homolog (*hcp-3*-like) genes (Supplementary Data S1) and their syntenic location (surrounding genes) (Figure 3.1). Core histone H3 and H3 variant genes were also obtained in these analyses but were easily distinguished from *hcp-3* homologs because of their high similarity to each other. Since our focus was on putative *hcp-3* orthologs and paralogs, we ignored both highly conserved core histone H3 and H3 variant proteins, as well as species-specific instances of highly diverged H3-like genes such as *F20D6.9* (also referred to as *D6H3*) from *C. elegans* (Henikoff, et al. 2000; Delaney, et al. 2018).

Unlike in holocentric insects (Drinnenberg, et al. 2014), we found that *hcp-3* orthologs are strictly retained in all *Caenorhabditis* species. In 28 of 32 species, they are found in shared syntenic locations, between genes homologous to *C. elegans hlh-11* and *F58A4.6* (Figure 3.1). In three of the four remaining species, at least partial synteny is maintained downstream of *hcp-3* (genes *F58A4.6*, *pri-1*, and *bbs-4)* whereas upstream synteny is either not maintained (in *C. tropicalis*) or cannot be discerned due to short genomic scaffolds (*C. waitukubuli* and *C. japonica*, Figure 3.1). Only *C. species 49* (*C. sp49*) lacks an *hcp-3* gene in this shared syntenic locus. Based on its presence in the ancestral locus in its sister species *C. sp25* and all other species, we infer that this movement of *hcp-3* is specific to *C. sp49*. *C. sp49* encodes two *CenH3*

paralogs, both found in new syntenic loci that are not shared with sister species. We arbitrarily assign one homolog as *hcp-3* and the other as *hcp-3L9* (further explained below).

In addition to *hcp-3* orthologs, we found that thirteen out of thirty-two examined species encode at least one additional *hcp-3*-like sequence. We refer to these paralogs as "*hcp-3L*" genes (for *hcp-3 Like*) (Figure 3.1). These *hcp-3L* genes include previously reported *hcp-3* duplications in *C. remanei* and *C. elegans* (Monen, et al. 2005; Monen, et al. 2015), which we refer to as *hcp-3L4* and *cpar-1* (as previously named, also referred to as *hcp-3L1* in Figures 3.1 and 3.2), respectively. We also identified one additional *hcp-3L* paralog in *C. tribulationis*, *C. sp41, C. sinica*, *C. latens*, *C. brenneri*, *C. doughertyi*, *C. sp54*, *C. panamensis*, *C. afra,* and *C. sp49*, and two, independent *hcp-3L* paralogs in *C. sp48*. In most cases, *hcp-3L* paralogs shared identical exon-intron structure as their orthologs. However, we also observed a few instances of intron losses and gains in *hcp-3* or *hcp-3L* genes (Supplementary Figure 3.S1). Such partial intron losses have been observed previously in plants (Roy and Penny 2007), fungi (Nielsen, et al. 2004) and in *Caenorhabditis* species (Robertson 1998; Cho, et al. 2004; Kiontke, et al. 2004) and are thought to result from partial retrotransposition, in which cDNA partially replaced the genomic locus.

All *hcp-3* and *hcp-3L* genes encode proteins with conserved Histone Fold Domains (HFD) (See Supplementary Data), which are between 69-100% identical to the HFD of HCP-3 from the same species (Figure 3.1). In contrast, their N-terminal domains show high divergence from HCP-3 orthologs (26-97% identical, Figure 3.1). This pattern is consistent with overall trends of *CenH3* evolution, where the HFDs are more evolutionarily constrained due to

interactions with other histones, whereas the N-terminal domains can be so divergent that they cannot even be reliably aligned across different lineages (Malik and Henikoff 2001).

We next used a combination of syntenic and phylogenetic analyses to determine whether *hcp-3L* paralogs were shared between different species, which would indicate their functional co-retention with *hcp-3* orthologs for long evolutionary periods. The highly divergent N-terminal tail sequences of *hcp-3* and their paralogs cannot be reliably aligned and could distort our interpretations, so our phylogenies are based on HFD alignments. We first used the amino acid sequences for a maximum likelihood phylogenetic analysis (Supplementary Figure 3.S2). We found that the protein-based phylogeny suffered from poor resolution, was unable to resolve most of the important branches and groupings of interest and was even incongruous with the well-accepted *Caenorhadbitis* phylogeny. Therefore, we built a maximum likelihood phylogenetic tree using a codon-based alignment of the conserved HFD cDNA sequence (Figure 3.2). This phylogeny is much better resolved especially at shallow nodes (both phylogenies suffer from lack of resolution at deeply branching nodes) and largely agrees with our findings from the shared synteny analyses. For example, both synteny and phylogenetic analyses suggest that the duplication that gave rise to *hcp-3L4* occurred prior to the common ancestor of *C. latens* and *C. remanei* (Figure 3.2). Similarly, we can infer that *hcp-3L5* duplicated in the common ancestor of *C. sp48* and *C. brenneri*. In contrast, the *hcp-3L* paralogs in *C. doughertyi*, *C. sp54*, *C. elegans*, *C. panamensis, C. afra*, *C. sp49*, and the additional *hcp-3L* paralog in *C. sp48* each arose via seven independent duplications (Figure 3.2). In each of these seven species, the *hcp-3L* paralogs are present in unique genomic locations (Figure 3.1) and typically group most closely with *hcp-3* orthologs from the same species (Figure 3.2).

The only discrepancy between the synteny and phylogenetic analyses was for *hcp-3L2* genes found in *C. tribulationis, C. sp41* and *C. sinica*. These species are part of a group, with *C. sinica* believed to be an outgroup to *C. tribulationis, C. sp41,* and *C. 90anzibari*. Different genomic locations of *hcp-3L* duplicates among *C. tribulationis, C. sp41 and C. sinica* (Figure 3.1) would suggest that the duplications are the result of independent duplication events although the small size of *C. sinica* genomic scaffolds leave its shared synteny status ambiguous. In contrast, our phylogenetic analyses group *hcp-3L* genes from these species together with a high degree of confidence (Figure 3.2), suggesting that *hcp-3L2* is the result of a single duplication event, followed by transposition of this gene to a new locus in *C. sinica*. We infer that the absence of *hcp-3L2* in *C. zanzibari* could be the result of gene loss although there is no evidence of *hcp-3L* loss in any other species. Another possibility is that *C. zanzibari* may be ancestral to *C. tribulationis* and *C. sinica* for the *hcp-3L* syntenic location, in contrast to the accepted species phylogeny, and may have never acquired a *hcp-3L* paralog. Recent studies have revealed widespread roles in diverse taxa for introgression and/or incomplete lineage sorting, leading to different genomic locations having vastly different evolutionary histories (Hobolth, et al. 2011; Mailund, et al. 2014; Ginsberg, et al. 2019; Suvorov, et al. 2022). Thus, it is formally possible that *C. zanzibari* never acquired *hcp-3L2.* However, based on the well-resolved species phylogeny of this quartet of species, we favor the first possibility that *C. zanzibari* acquired, then lost *hcp-3L2.* Therefore, our analyses reveal that *hcp-3* has duplicated at least ten independent times within *Caenorhabditis* species.

We examined the expression of *hcp-3* and *hcp-3L* genes across representative *Caenorhabditis* species. We used RT-PCR analyses using specific primers on template RNA collected from a mixed population of males and females or hermaphrodites at various larval stages (see Methods). All analyzed species expressed both ancestral and duplicate *hcp-3* genes (Figure 3.3, Supplementary Figure 3.S3). We investigated whether *Caenorhabditis hcp-3L* genes have sex-restricted expression as is seen in some *Drosophila CenH3* paralogs (Kursel and Malik 2017). We performed RT-PCR on RNA collected from L4/young adult males or from L4/young adult hermaphrodites or females (these developmental stages capture both female and male meiosis). Unlike *Drosophila CenH3* paralogs, we did not find sex-restricted expression of any *hcp-3L* genes (Figure 3.3, Supplementary Figure 3.S3); instead, they appear to be expressed in both sexes.

3.2.2 Motif retention and loss in the N-terminal region of HCP-3 and HCP-3L proteins

Although CenH3 proteins all have a relatively conserved HFD, their N-terminal tails are often so divergent that they cannot be aligned nor even be considered homologous across different lineages (Malik and Henikoff 2001). Nevertheless, conserved motifs have been identified in the N-terminal tails of CenH3 proteins from many other lineages including *Drosophila*, mosquitos, and plants using alignment-independent approaches (Maheshwari, et al. 2015; Kursel and Malik 2017; Kursel, et al. 2020). These N-terminal tail motifs are often highly conserved within a lineage, but not conserved across different lineages. Although no such studies have been previously performed for the *Caenorhabditis* HCP-3 proteins, recent studies show that the N-terminal tail of *C. elegans* HCP-3 interacts with the inner kinetochore protein KNL-2 via a predicted structured region (de Groot, et al. 2021; Prosée, et al. 2021). This interaction between

KNL-2 and HCP-3 is necessary for the establishment of centromeres in the hermaphrodite germline, prior to the first embryonic mitosis (Prosée, et al. 2021).

We took advantage of our comprehensive identification of HCP-3 and HCP-3L proteins to *de novo* identify conserved residues or motifs in their N-terminal tails using the MEME suite of software (Bailey, et al. 2015) as previously described (Kursel and Malik 2017) (see Methods). For this, we first identified motifs by analyzing all *Caenorhabditis* species encoding a single HCP-3 protein, which are more likely to have retained all motifs essential for their functions. Using this analysis, we identified 13 motifs within HCP-3 (Figure 3.4A, Supplementary Figure 3.S4), numbered sequentially from the N-terminus, with 11 motifs in the N-terminal tail and motifs 12 and 13 in the histone fold domain (HFD). Not all 13 motifs are universally present in species encoding a single *hcp-3* gene. For example, motif 2 is present in only a subset of species examined. Based on phylogenetic analyses, we infer that motif 2 was acquired in the ancestor of a clade of eight species which includes *C. sulstoni* and *C. becei* (Figure 3.4B).

In the second step, we investigated how well these 13 motifs are conserved in species containing *hcp-3L* genes. We found that these motifs varied in their evolutionary stability and conservation. N-terminal tail motifs 1-11 are more variably retained than HFD motifs 12-13 (Supplementary Figure 3.S4), which are present in all HCP-3 and HCP-3L proteins as expected, except for HCP-3L3 from *C. sp54,* which has a divergent HFD. Overall, the motifs we have defined account for 48% and 63% of the total N-tail sequence in *C. elegans* HCP-3 and CPAR-1, respectively.

Our initial unsupervised motif analysis found that motif 3 was universally conserved in all HCP-3 and HCP-3L proteins (Figure 3.4B). In contrast, motifs 1 and 4 were universally retained in at least one paralog in each species (often both) with only a few exceptions. Recognizing that apparent 'motif loss' might be the result of indels or divergence of a critical conserved residue, we manually re-examined the sequences missing either motif 1 or 4 to see if they were missed because they fell below the statistical threshold of the unsupervised motif analysis. Based on these analyses (Supplementary Data File S4), we were able to confirm the presence of motifs 1 and 4 in all species (Figure 3.4B). Thus, three motifs (1, 3, and 4) are present in at least one HCP-3 paralog in all species. Notably, these motifs have not been identified in previous analyses of the N-terminal tail, highlighting the value of alignment-independent methods. These motifs include residues that are almost universally conserved in *Caenorhabditis* species (asterisks in Figure 3.4A). We predict that mutation of these residues may reveal important insight about the various functions of the HCP-3 N-terminal tail, including its interactions with kinetochore proteins such as KNL-2.

Motifs 5, 6, 7, 9, and 11 were less conserved, being present in 78-94% of species. For example, motif 6 appears to be lost in both HCP-3 paralogs from *C. tribulationis* and *C. afra*, while motif 11 is not found in *C. wallacei*, *C. elegans* (both paralogs), and in sister species *C. sulstoni* and *C. afra* (both paralogs). Motif 5 includes a 4-amino acid segment, ExxR (Figure 3.4A, where x represents any amino acid) that constitutes a putative cleavage motif for the separase enzyme, which initiates anaphase by cleaving the kleisin subunit of cohesin (Monen, et al. 2015). Although this ExxR motif is found in both HCP-3 and CPAR-1 in *C. elegans,* only the latter is cleaved by separase (Monen, et al. 2015). This suggested that the ExxR motif is

necessary but not sufficient for efficient separase cleavage. Since CPAR-1 is not associated with centromeres (Gassmann, et al. 2012; Monen, et al. 2015), it is difficult to establish the significance of the ExxR motif, whose mutation led to no deleterious fitness consequences (Monen, et al. 2015). In cases where motif 5 was missing, individual alignments of HCP-3 sequences allowed us to identify the ExxR separase motif in all HCP-3 and HCP-3L proteins, except for HCP-3L proteins from *C. latens* and *C. sp48* (Supplementary Figure 3.S5). Thus, although it is unclear whether it is required for separase cleavage or some other function, the ExxR motif is nevertheless largely conserved in all HCP-3 proteins and most HCP-3L proteins. It is possible that the cleavage site mediates the removal of the N-terminal tail from certain HCP-3L proteins, thereby eliminating it from a role in germline re-establishment of centromere identity (Prosée, et al. 2021).

Even though motif 2 was only acquired in eight *Caenorhabditis* species (Figure 3.4B), it has been retained in at least one HCP-3 paralog of each of these species. The only instances of motif 2 loss are seen in *C. panamensis hcp-3L10* and in *C. afra hcp-3*. Our findings suggest that motif 2 is functionally important in these species despite not being universally present within all *Caenorhabditis* species. We hypothesize that some clade-specific HCP-3 protein-protein interactions or functions were acquired via motif 2 in the ancestor of these eight species.

In some instances, motif loss occurred in only one of the two HCP-3 paralogs from the same species. For example, most HCP-3L proteins lack motif 7 whereas ancestral HCP-3 in the same species usually contained this motif. Similarly, in species containing motif 5 and/or 6, the *hcp-3L* gene almost always lost these motifs whereas the ancestral *hcp-3* maintained them. In

sister species *C. brenneri* and *C. sp48,* the converse is seen, where motif 11 is maintained in the duplicate *hcp-3L* gene but lost in ancestral *hcp-3*. Overall, however, motif loss tends to occur more frequently in the *hcp-3L* paralog instead of the ancestral *hcp-3*. Thus, *hcp-3L* paralogs may be capable of performing only a subset of the functions of an ancestral *hcp-3*. This asymmetric pattern of motif loss may also explain why ancestral *hcp-3* has been universally retained in all *Caenorhabditis* species, whereas *hcp-3L* paralogs are rarely present in more than two species.

3.2.3 Selective constraints on hcp-3 orthologs and hcp-3L paralogs

Our study represents an opportunity to evaluate the selective pressures imposed on *CenH3* genes either due to holocentricity or due to their recurrent duplication. A previous analysis had concluded there was weak evidence of positive selection from an analysis of *hcp-3* sequences from six divergent *Caenorhabditis* species whose sequence was available at that time (Zedek and Bureš 2012). However, extremely large divergence and low number of sequences can result in false signals of positive selection. Therefore, we revisited this analysis using maximum likelihood methods (see Methods). We separately analyzed *hcp-3* sequences from the two deep lineages of *Caenorhabditis* species evaluated here, as well as two subsets of species from one of the lineages for which we had enough representation (Supplemental Table S1A). In every case, we found no evidence of positive selection acting on *hcp-3* genes.

Since the presence of a paralog within the genome may affect the selective constraint on the ancestral *hcp-3* gene, we repeated the analysis by intentionally excluding all species that encode one or more *hcp-3L* paralogs (Supplemental Table S1A). Once again, we found no evidence for positive selection. Thus, in contrast to the previous study (Zedek and Bureš 2012)

and in contrast to findings that *CenH3* genes from multiple other animal and plant taxa evolve under positive selection (Malik and Henikoff 2001; Talbert, et al. 2004; Schueler, et al. 2010; Finseth, et al. 2015), we find no evidence for positive selection acting on *CenH3* genes in *Caenorhabditis*. Our inability to detect positive selection may reflect a lack of statistical power, although we note that the tree lengths used in our analysis are highly appropriate for such analyses.

Based on their presence in few species, we infer that most of the *hcp-3L* genes we identified in *Caenorhabditis* species are relatively young. Our finding that *hcp-3L* genes bore the brunt of motif loss (Figure 3.4) raised the possibility that many *hcp-3L* genes are not functionally constrained. To address this possibility, we carried out three types of analyses. First, we examined selective constraints acting on *hcp-3* and *cpar-1* by investigating polymorphisms within natural isolates of *C. elegans* strains that have been previously sequenced (Cook, et al. 2017) (Supplementary Figure 3.S6). We found only three synonymous (amino acid preserving) and zero non-synonymous (amino acid altering) polymorphisms in *hcp-3*. In contrast, *cpar-1* contained two synonymous polymorphisms (including one commonly shared between more than 25 strains) and six non-synonymous polymorphisms, four of which are shared among more than seven *C. elegans* strains. Some of these polymorphisms arise in otherwise conserved positions in the N-terminal tail (Figure 3.4, Supplementary Figure 3.S6) or HFD, implying that they are likely deleterious for function. In addition to non-synonymous changes, we found at least two strains that may have disrupted *cpar-1*, via either a frameshift or a splice site mutation. Based on this comparison, we infer that *cpar-1* is evolving under lower functional constraints than *hcp-3* in *C. elegans*.

Second, we tested whether *hcp-3L* paralogs are generally evolving under fewer stringent functional constraints than *hcp-3* genes. For this, we calculated dN/dS values, which measure the ratio of the normalized rates of non-synonymous substitutions to synonymous substitutions. A lower dN/dS ratio is reflective of higher functional constraints, whereas a dN/dS ratio of close to 1 is reflective of lack of functional constraints for protein-coding function. We calculated dN/dS values in pairwise comparisons of the HFD of *hcp-3L* orthologs present in two distinct species: *hcp-3L4* in *C. latens* and *C. remanei, hcp-3L2* in *C. sinica* and *C. tribulationis,* and *hcp-3L5 in C. brenneri* and *C. sp48* (Supplemental Table 1B). We obtained dN/dS ratios of 0.02, 0.04, and 0.08, respectively. These values are considerably lower than 1, suggesting that all three paralogs have been retained under functional constraint for protein-coding function during the divergence of the respective *Caenorhabditis* species. Moreover, in all three cases, we found that dN/dS values for *hcp-3L* orthologs were comparable to or lower than corresponding *hcp-3* orthologs from the same species (Supplemental Table 1B). For comparison, the dN/dS values for pairwise comparisons of ancestral *hcp-3* from *C. latens/ C. remanei*, *C. sinica/ C. tribulationis* and *C. brenneri/ C. sp48* are 0.18, 0.02 and 0.03, respectively. Thus, unlike *cpar-1* in *C. elegans*, we find that *hcp-3L* paralogs have evolved under similar or even more stringent constraints than ancestral *hcp-3* genes at least in some *Caenorhabditis* species.

Given this finding, we revisited the age of the *hcp-3L* paralogs in *Caenorhabditis* species in a third analysis. Unlike dN or dN/dS values, dS values are relatively unaffected by selective constraints and provide a more reliable proxy for their divergence from *hcp-3* ancestors. We calculated the synonymous divergence (dS) between the HFD of *hcp-3L* paralogs whose closest

relatives are *hcp-3* orthologs from the same species (Figure 3.2). These dS values range from 0.15 (for *C. afra*) to 0.74 (for *C. doughertyi*) (Supplemental Table 1B). These dS values are considerably lower than seen between *Drosophila CenH3* paralogs in the same species (*e.g., D. virilis*). Although we lack reliable molecular clock-like estimates to convert these dS values to millions of years of divergence (Cutter 2008), the dS values are high enough to imply that a majority of these *hcp-3L* paralogs have been functionally retained for several million years, even though most of them have not been retained across multiple speciation events (Figure 3.1).

The overall selective pressure acting on *hcp-3L* paralogs is that of purifying selection or evolutionary constraint. However, our comparison of HFD between *hcp-3* and *hcp-3L3* from *C. sp54* revealed a dN/dS of 1.74 in a maximum likelihood test, although this is not statistically significantly different from the neutral expectation of dN/dS =1. Based on the phylogeny of *CenH3* HFD (Figure 3.2), we could infer that *C. sp44 hcp-3* is an outgroup to the two *C. sp54 hcp-3* genes. We compared *C. sp44 hcp-3* to either *hcp-3* or *hcp-3L3* from *C. sp54*. These analyses revealed a lower dN/dS in a comparison between the two ancestral *hcp-3* orthologs (dN/dS =0.09) than between *C. sp44 hcp-3* and *C. sp54 hcp-3L3* (dN/dS = 0.34). This implies that it is the unusual paralog, *hcp-3L3*, that has evolved more rapidly. This combined with our finding that HCP-3L3 contains duplications of the N-terminal tail motifs (Figure 3.5A) suggests the possibility of incipient neofunctionalization of the *hcp-3L3* paralog in *C. sp54*.

3.2.4 Duplication of other centromere-localized proteins in Caenorhabditis species

In most cases, the protein sequence of HCP-3 paralogs can be confidently aligned to the ancestral HCP-3, indicating clear homology. However, aligning *C. afra* HCP-3 and *C. afra* HCP-

3L8 revealed that the paralog contained an additional 198 amino acids on its N-terminus. This region was not homologous to HCP-3. To our surprise, we found that this segment was instead homologous to CENP-C (known as HCP-4 in *C. elegans*). HCP-4 and HCP-3 directly interact with each other in *C. elegans* (Oegema, et al. 2001) and in other eukaryotes. *C. afra hcp-3L8* contained two copies of *C. afra hcp-4* exons 1 and 2, followed by a partial copy of *hcp-4* exon 3. These *hcp-4* homologous segments are contiguous with *hcp-3*-homologous sequence to constitute the *hcp-3L8* coding sequence (Figure 3.5B). We used RT-PCR to confirm that *hcp-3L8* was transcribed as a single transcript containing homology to both *hcp-4* and *hcp-3* sequences (Figure 3.5C). Therefore, *C. afra hcp-3L8* is a chimera of *hcp-4* and *hcp-3*. In addition to this *hcp-4-hcp-3* fusion gene, *C. afra* also maintains its ancestral *hcp-3* and *hcp-4* genes. The functional roles of the HCP-4-like regions present within *hcp-3L8* are unknown. However, a conserved CENP-C motif is absent in this chimera. The conserved CENP-C motif, which mediates the interaction with the CenH3 nucleosome (Kato, et al. 2013), is present at the C-terminus of *C. elegans* HCP-4 (Moore and Roth 2001). Thus, loss of the CENP-C motif in HCP-3L8 is not unexpected since the HCP-4 and HCP-3 segments are already physically linked to each other in this chimeric protein.

Encouraged by this finding of *hcp-4* duplication and fusion with *hcp-3* in *C. afra*, we investigated whether other centromere-localized proteins have also duplicated and diversified like *hcp-3*. We performed similar paralog searches for proteins from the inner kinetochore (*hcp-4* and *knl-2*), middle kinetochore (*knl-1*), and outer kinetochore (*him-10*, *ndc-80*, *spdl-1*, and *zwl-1*). We found an intact copy of each ancestral gene in every species (Figure 3.6) except for two instances where we were unable to identify full-length intact *zwl-1* genes (in *C. kamaaina* and *C.*

tropicalis) ('#' in Figure 3.6). We found instances of duplications for all kinetochore proteins except *zwl-1*. These duplications either appear to be retained with an intact open reading frame (filled, gray arrows), or are interrupted (double lines), or show clear signs of pseudogenization (unfilled arrows) (Figure 3.6). In the 32 species examined, we found seven *hcp-4* duplicates, four *knl-2* duplicates (including a pseudogene in *C. brenneri*), eight *knl-1* duplicates, four *spdl-1* duplicates, five *ndc-80* duplicates (including two pseudogenes), and three *him-10* duplicates (including one pseudogene). Duplications of inner and middle kinetochore proteins were only marginally more prevalent than duplications of outer kinetochore proteins. Interestingly, we observed several instances of partial intron losses that occurred recurrently in genes encoding ancestral and paralog outer kinetochore proteins (Supplementary Figure 3.S7) like what we observed previously for *hcp-3* and *hcp-3L* genes (Supplementary Figure 3.S1). Overall, our analyses suggest that in addition to HCP-3, other kinetochore proteins are also undergoing duplication and diversification in *Caenorhabditis* species.

To understand the evolutionary constraints on *Caenorhabditis* kinetochore proteins, we analyzed these genes using maximum likelihood methods. We found no evidence of positive selection acting on ancestral *hcp-4*, *knl-1*, *knl-2*, *zwl-1*, *spdl-1*, *ndc-80,* and *him-10* genes in either the *C. elegans* or *C. afra* clades (Supplemental Table S1C). Next, we examined the evolutionary constraints acting on paralogs of kinetochore proteins by comparing the paralogs to the ancestral kinetochore genes from the same species. In all cases except two, we found strong evidence that that the duplicates are retained under strong purifying selection (Supplemental Table S1D). For *knl-2* in *C. inopinata* and *ndc-80* in *C. sp54*, we could not rule out the null hypothesis of neutral evolution.

We investigated whether any kinetochore protein paralogs have been co-retained with *hcp-3L* paralogs, which would suggest a concerted duplication and retention of multiple kinetochore proteins, consistent with significant specialization. We found that four of six independent *hcp-4* duplications coincided with retention of *hcp-3L* paralogs in the same species (Figure 3.6). These include an *hcp-4* paralog whose origin coincides with the *hcp-3L4* paralog in *C. latens* and *C. remanei*, two *hcp-4* paralogs that co-occur with *hcp-3L3* in *C. sp54*, and the *hcp-4-hcp-3* fusion gene in *C. afra* (*hcp-3L8*). Thus, 4 of 14 species containing an *hcp-3L* paralog also encode a (complete or partial) *hcp-4* paralog whereas 2 of 18 species lacking *hcp-3L* paralogs encode a *hcp-4* paralog: *C. sp44* and *C. kamaaina.* Thus, there is no statistically significant evidence of co-retention (p=0.36), indicating that the duplication or retention of *hcp-3* and *hcp-4* paralogs may be independent.

Other kinetochore proteins analyzed also largely reflect this pattern of independent duplication. Even though KNL-2 is required to deposit HCP-3 proteins at centromeres in *Caenorhabditis* species (Maddox, et al. 2007; de Groot, et al. 2021; Prosée, et al. 2021), there does not appear to be a significant pattern of co-retention with *hcp-3L* paralogs. The one exceptional species is *C. sp54*, which encodes an *hcp-3L3* paralog, two *hcp-4* paralogs, a *knl-2* paralog, a *knl-1* paralog, an *ndc-80* paralog, and a *him-10* paralog. If the proteins encoded by these paralogs exclusively interact with each other, this species may represent an intriguing case of incipient kinetochore specialization.

3.3 DISCUSSION

Our analyses reveal that *hcp-3* has duplicated at least ten independent times within *Caenorhabditis* species. In contrast to ancient co-retention of *CenH3* paralogs in plants, *Drosophila*, mosquito species (Maheshwari, et al. 2015; Kursel and Malik 2017; Kursel, et al. 2020; Kursel, et al. 2021), and even holocentric *Meloidogyne* nematode species (Despot-Slade, et al. 2021), we observed only a few cases of *hcp-3L* paralogs that are shared across two or three *Caenorhabditis* sister species, although this may partly reflect density of species sampling in these different taxonomic groups. Our findings suggest that most of the *hcp-3L* paralogs we have found are relatively young, assuming the relative ages of *Caenorhabditis* and *Drosophila* species analyzed are comparable (Cutter 2008).

Our comprehensive phylogenomic approach in *Caenorhabditis* nematodes uncovered two novel aspects of CenH3 evolution. First, we uncovered a detailed molecular architecture of the N-terminal tail of HCP-3 proteins (Figure 3.4). The HCP-3 N-terminal tail is dispensable for mitotic chromosome segregation and centromere maintenance during *C. elegans* development (Prosée, et al. 2021) but is essential in establishing a functional HCP-3 distribution in the germline, which is maintained in the subsequent generation throughout development. At least part of this functionality of the HCP-3 N-terminal tail stems from its interactions with kinetochore proteins like KNL-2 (de Groot, et al. 2021; Prosée, et al. 2021). Thus far, however, the molecular architecture of the interactions of HCP-3 with other kinetochore proteins like KNL-2 has been only crudely defined. Like in other eukaryotic lineages, the N-terminal tail of HCP-3 proteins is much more divergent than the histone fold domain (HFD). Thus, comparisons of functional domains in CenH3 N-terminal tails between taxonomic groups or even within *Caenorhabditis* are very difficult, exacerbating the difficulty in defining functional domains

within HCP-3's N-terminal tail. Our description of 11 motifs in HCP-3 N-terminal tails, including three that are nearly universally conserved, provides an important resource for the fine-scale dissection of the various protein-protein interactions mediated by the N-terminal tail and the functional role these interactions play in centromere biology. In particular, the three conserved motifs contain residues that are as well conserved as many HFD residues across *Caenorhabditis* species.

We propose that these N-terminal tail motifs are sites of previously proposed or novel protein-protein interactions, either with kinetochore proteins or with other chromatin factors that could intersect with holocentromere formation or maintenance. Consequently, motif gains or losses could indicate gains or losses of HCP-3 interactions with partner proteins. We observe one unambiguous case of motif gain in one clade of *Caenorhabditis* species. Motif 2 likely represents a novel protein-protein interaction module important for *CenH3* function at least in those species. We also observe several cases of motif degeneration or loss. Unlike in *Drosophila CenH3* paralogs (Kursel and Malik 2017), we see little evidence for motif redistribution between the paralog and ancestral *hcp-3* genes, which would suggest sub-functionalization; the only exception is motif 2 that appears to be present in either HCP-3 or HCP-3L proteins, but not both. Overall, we find that motif loss or degeneration preferentially occurs in *hcp-3L* paralogs rather than ancestral *hcp-3*, suggesting that the paralogs progressively lose ancestral functions and interactions. Since tail-less HCP-3 proteins can still function in mitosis (Prosée, et al. 2021), it is tempting to speculate that HCP-3L paralogs could still function in mitosis despite progressive loss of N-terminal motifs.

The remarkable example of a chimeric gene in *C. afra,* where an HCP-3L protein is fused to an inner kinetochore protein, HCP-4 (CENP-C in mammals; Figure 3.5) exemplifies an instance where previously conserved motifs could be lost. HCP-3 and HCP-4 physically interact in many eukaryotes to form the kinetochore complex during mitosis. The fusion of these two proteins in HCP-3L8 guarantees a protein-protein interaction, which is consistent with the loss of the CENP-C motif (Kato, et al. 2013) required for HCP-3L and HCP-4 interactions. This could also lead to loss of HCP-3L N-terminal tail motifs required for HCP-4 association.

The second major conclusion from our evolutionary analyses is the unusually rapid cadence of turnover of *hcp-3* paralogs in *Caenorhabditis* species. Nearly half of the species we analyzed contain an *hcp-3* paralog. Yet, in contrast to analyses in *Drosophila* and mosquito lineages, where duplicates were older and fewer in number (Kursel and Malik 2017; Kursel, et al. 2020), the *Caenorhabditis* paralogs were acquired through ten independent duplication events. Most paralogs have only been retained in a single species with only one *hcp-3L* paralog being present in more than two species. Previous analyses suggest that *C. elegans* have a higher gene duplication rate than other species including *D. melanogaster* (Lynch and Conery 2000; Pan and Zhang 2007; Lipinski, et al. 2011), potentially as high a duplication rate per gene as 0.02 every million years (Lynch and Conery 2000). This high rate of gene duplication may account for the higher number of *hcp-3* duplications we observe in *Caenorhabditis.* However, these analyses also suggest that the vast majority of gene duplications that arise in *C. elegans* are efficiently purged by natural selection (Lipinski, et al. 2011). In contrast, our findings suggest that many *hcp-3L* paralogs are retained under purifying selection for significant periods of time.

Our evolutionary analyses thus reveal an unusual 'revolving-door' of *hcp-3L* paralogs in *Caenorhabditis* species. Under this regime, gene duplication is frequent, *hcp-3L* paralogs are retained under purifying selection for a significant evolutionary period before eventually either degenerating (*e.g.*, possibly *cpar-1* in *C. elegans*) or being lost entirely (*e.g.*, possibly *hcp-3L2* in *C. zanzibari*), returning to the ancestral state of the genome encoding only a single *hcp-3* gene. This cadence is unprecedented among most other taxonomic groups where *CenH3* duplications have been investigated. Even the high number of *hcp-3* duplications we have observed is likely an under-estimate of the true number, since extant species represent only one evolutionary snapshot. Indeed, our study implies that many previously arising *hcp-3L* paralogs have been lost or degenerated beyond recognition during *Caenorhabditis* evolution. This is akin to the 'revolving door' of HP1-family proteins previously reported in *Drosophila* (Levine, et al. 2012). Although we have not evaluated all of them in the same level of detail, duplications of other kinetochore proteins in *Caenorhabditis* also appear to occur with a similar revolving-door dynamic.

What could account for this revolving-door, *i.e.*, the short-term evolutionary retention of *hcp-3L* paralogs and their long-term loss or degeneration? We consider several possibilities for the sources of transient selective pressure to retain *CenH3* paralogs. First, this pattern could result from specialization of kinetochore paralogs for functions that are unrelated to chromosome segregation, as has been recently shown in *Caenorhabditis* and *Drosophila* neurodevelopment (Cheerambathur, et al. 2019; Zhao, et al. 2019). A previous study showed that CPAR-1 localizes to chromosomes but not centromeres in *C. elegans* (Monen, et al. 2015), although it is unclear whether this is typical for other HCP-3L paralogs.

A second explanation for this pattern might be sub-functionalization of *CenH3* paralogs for tissue- or sex-specific or meiosis-specific functions, as is proposed in *Drosophila* species (Despot-Slade, et al. 2021; Kursel, et al. 2021). Unlike monocentric chromosomes, holocentric chromosomes experience inherent challenges during meiosis, which have been overcome in different taxa via different means (Melters, et al. 2012; Marques and Pedrosa-Harand 2016). A recent study In *Meloidogyne* nematode species found that an ancestral aCenH3 is deeply conserved for function in mitosis whereas more rapidly evolving CenH3 paralogs lost mitotic function (Despot-Slade, et al. 2021). However, we found no evidence of sex-specific expression of *CenH3* paralogs in *Caenorhabditis* species. Moreover, unlike in most eukaryotes, *C. elegans* chromosomes connect to the meiotic spindle by a CenH3-independent mechanism (Monen, et al. 2005). Therefore, at least in *C. elegans*, *hcp-3* is entirely dispensable for meiotic chromosome segregation (Monen, et al. 2005). This relaxes constraints to maintain meiotic functions on *hcp-3* genes but cannot explain the revolving-door pattern.

A third possible explanation for the transient retention of *hcp-3* paralogs is suppression of either 'centromere-drive' or 'holokinetic drive'. Currently, it is unclear whether centromere drive could occur in holocentric organisms (Zedek and Bureš 2012, 2016; Krátká, et al. 2021). Although a previous study reported weak evidence of positive selection using an analysis of *hcp-3* from six highly diverged *Caenorhabditis* species (Zedek and Bureš 2012), our comprehensive reanalysis of *hcp-3* evolution across a much more densely-sampled series of closely-related species revealed no evidence of positive selection (Supplemental Table S1B). Similarly, the *aCenH3* gene required for mitosis is deeply conserved and slowly evolving in *Meloidogyne*

nematodes although other *CenH3* paralogs appear to be rapidly evolving (Despot-Slade, et al. 2021). Asymmetric meiosis in nematode species could also lead to another form of drive, leading to preferential inheritance of larger or smaller holocentric chromosomes ('holokinetic drive'), which could explain the observed negative correlation between chromosome number and genome size in many holocentric lineages (Bureš and Zedek 2014). If either of these drive mechanisms occur in *Caenorhabditis* species, then *hcp-3L* paralogs could arise and be temporarily retained as drive-suppressors, but only while the driving elements were still present in the genome. This suppression might result in loss of these driving elements from the genome, rendering *hcp-3L* gene functions superfluous and resulting in subsequent loss of these paralogs. Given the uncertainty about the existence of centromere-drive or holokinetic drive in nematodes, or the role that *hcp-3L* paralogs might play in either process, we cannot elaborate further on this possibility.

We favor a fourth hypothesis, in which the holocentricity of *Caenorhabditis* species, with HCP-3 distributed along the length of the chromosomes, might itself lead to the revolving-door dynamics of centromeric proteins. CenH3 incorporation into nucleosomes at holocentromeres is more plastic than at monocentromeres. Since CenH3 does not have to associate with specific sequences or chromosomal regions, holocentric chromosomes more easily tolerate chromosome breakage, fusion, or rearrangements. Indeed, even prior to clear cytological evidence, holocentric organisms were observed to maintain fertility despite radiation-induced chromosome breaks (Schrader 1935; Melters, et al. 2012). Moreover, even completely foreign DNA can form mini-chromosomes that assemble centromeres and be stably propagated (Zhu, et al. 2018; Lin and Yuen 2020; Lin, et al. 2021). Nevertheless, centromere distribution in holocentric organisms is

not random. Although HCP-3 presence is partially linked to certain 'HOT (High Occupancy Target) sites' in *C. elegans* (Steiner and Henikoff 2014), the overall pattern of centromere establishment in *C. elegans* appears to be predominantly linked to transcriptionally repressed genomic regions in the germline. This pattern of centromere definition via transcriptional inactivity is seen in both *C. elegans* and in the CenH3-devoid *Bombyx mori* (Gassmann, et al. 2012; Steiner and Henikoff 2014; Senaratne, et al. 2021). In contrast, some holocentric species like *Meloidogyne* nematodes and *Rhynchospora* plants localize their CenH3 proteins to specific repeats found distributed over the genome (Marques, et al. 2015; Despot-Slade, et al. 2021; Hofstatter, et al. 2022).

Although a transcriptional quiescence-dependent mode of centromere definition is more tolerant of genomic rearrangements than monocentric organisms, it could also be subject to transient stress. This stress could be imposed by either chromosomal rearrangements or transposon invasion, which can quickly and dramatically alter the landscape of transcription and repression in the germline. In such circumstances, it might be advantageous to retain HCP-3L paralogs to temporarily increase the dosage of proteins required to correctly establish centromere identity, as has been proposed in some plant lineages (Evtushenko, et al. 2021). Alternatively, it may be advantageous to express HCP-3 proteins with slightly altered sequences and localization preferences, allowing restoration of optimal centromere distributions even after periods of such 'genomic stress'. Under either scenario, eventual amelioration of the genomic stressor (*e.g.*, decay or silencing of the invading transposable element) would render *hcp-3L* paralogs superfluous and these would be lost. Therefore, we hypothesize that holocentric species like *C.*

elegans, which rely on a transcriptional quiescence-dependent mode of centromere definition, may be prone to revolving-door dynamics of their kinetochore proteins.

Different *Caenorhabditis* species might represent different stages of the revolving-door process for kinetochore proteins. Species like *C. sp54*, which possess paralogs of five of seven kinetochore genes investigated, may be actively selecting for the retention and function of these paralogs. In contrast, species like *C. elegans*, with a possibly nonessential *cpar-1* and no other kinetochore paralogs, may have already overcome the need for such innovation. We therefore predict that functional consequences of kinetochore paralog loss in different *Caenorhabditis* species will differ based on their stage of genetic innovation. Our study underlines the need for the analysis of non-model organisms and the value of evolutionary comparisons to reveal novelties even in well-studied cellular pathways.

3.4 METHODS

3.4.1 Strain maintenance

All strains were cultured on Nematode Growth Medium (NGM) plates seeded with 200 µl OP50 at 20°C using standard methods (Brenner 1974).

3.4.2 Strains used

N2 *C. elegans*

DF5081 *C. japonica*

JU727 *C. sinica*

JU1333 *C. doughertyi*

JU2744 *C. tribulationis*

JU1199 *C. afra*

VX88 *C. latens*

QG702 *C. panamensis*

3.4.3 Identification of hcp-3 and kinetochore protein homologs in sequenced genomes

To identify *hcp-3* paralogs and orthologs we iteratively queried the assembled genomes of thirty-two *Caenorhabditis* species: *C. tribulationis*, *C. sp41*, *C. zanzibari*, *C. sinica*, *C. nigoni*, *C. briggsae*, *C. remanei*, *C. latens, C. sp51*, *C. sp44*, *C. sp48*, *C. brenneri*, *C. wallacei, C. tropicalis*, *C. doughteryi*, *C. sp54*, *C. inopinata*, *C. elegans, C. oiwi*, *C. kamaaina*, *C. waitukubuli*, *C. panamensis*, *C. nouraguensis*, *C. becei*, *C. yunquensis*, *C. macrosperma*, *C. sulstoni*, *C. afra, C. sp49*, *C. sp25*, *C. imperialis*, and *C. japonica* (Supplementary Data S2). We used tBLASTn (Altschul, et al. 1990; Altschul, et al. 1997) on each species' genome (Stevens, et al. 2019) to perform a homology-based search starting with *C. elegans* HCP-3 (WBGene00001831) as our query. We used a combination of gene predictions, publicly available RNA sequencing data, *hcp-3* alignments, and splice site predictions to annotate intron-exon regions of all *hcp-3* genes that were found. To ensure that we had not missed any *hcp-3* paralogs, we repeated our analyses querying each species' hits on their own genome using tBLASTn and did not retrieve additional hits. To identify paralogs and orthologs of kinetochore proteins (Figure 3.6), we repeated this same homology-search procedure starting with *C. elegans* HCP-4 (WBGene00001832), KNL-1 (WBGene00002231), KNL-2 (WBGene00019432), ZWL-1 (WBGene00021460), SPDL-1 (WBGene00015515), NDC-80 (WBGene00003576), and HIM-10 (WBGene00001869). We used http://blast.caenorhabditis.org/ to perform all tBLASTn analyses

using pre-set parameters and setting a e-value threshold of at least 10^{-1} to obtain all possible paralogs.

Synteny was used to determine *hcp-3* orthology across *Caenorhabditis* species. We identified annotated genes immediately upstream and downstream of *hcp-3* and *hcp-3L* genes. We then used these neighbouring genes as queries for tBLASTn searches of the *C. elegans* genome to identify the orthologous syntenic genes (Figure 3.1). Dissimilar flanking genes for different *hcp-3L* paralogs provide support for the phylogenetic inference that they were acquired through independent *hcp-3* duplication events. In some cases, *hcp-3 or hcp-3L* genes were found in small genomic scaffolds or at the end of scaffolds, reducing our ability to identify upstream or downstream syntenic genes. In the latter case, we analyzed additional genes in the direction (upstream or downstream) that had sufficient genomic information available on the same scaffold. Absence of *hcp-3* in the ancestral locus in *C. sp49 hcp-3* and of *hcp-3L2* in the duplicate locus in *C. zanzibari* was confirmed by using tBLASTn of each gene in the expected locus, resulting in no detectable homologous gene sequence.

3.4.4 Phylogenetic Analyses

All protein alignments were performed using the MUSCLE algorithm (Edgar 2004) in Geneious Prime 2019.2.3 (https://www.geneious.com). Codon-based nucleotide alignments were created using the MUSCLE (codon) feature in MEGAX (Kumar, et al. 2018). We used only the HFD for phylogenetic inference and used the maximum likelihood method implemented in MEGA11 (Stecher, et al. 2020; Tamura, et al. 2021). Our amino acid-based phylogeny used the JTT model (Jones, et al. 1992) and our nucleotide-based phylogeny used the General Time Reversible model (Nei and Kumar 2000). We inferred the bootstrap consensus tree from 100

replicates. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.8943)). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 26.05% sites). All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There was a total of 267 nucleotide positions in the final dataset between all HCP-3 and HCP-3L HFD amino acid sequences. Supplementary Tables 4C and 4D present the pairwise distances and number of differences, respectively, between all *hcp-3* and *hcp-3L* HFD coding sequences.

3.4.5 Motif Analyses

13 motifs were identified using MEME (Bailey, et al. 2015) on predicted, full-length HCP-3 protein sequences from species lacking *hcp-3* paralogs (*C. zanzibari, C. nigoni, C. briggsae, C. sp51, C. sp44, C. wallacei, C. tropicalis, C. inopinata, C. oiwi, C. kamaaina, C. waitukubuli, C. nouraguensis, C. becei, C. yunquensis, C. macrosperma, C. sulstoni, C. sp25, C. imperialis, C. japonica*). E-values of all 13 discovered motifs were below 10^{-5}. Motif logo plots were generated and downloaded from MEME. Presence or absence of these motifs in all HCP-3 and HCP-3L proteins was determined by using MAST (Bailey and Gribskov 1998). We considered a motif as present in a protein by using default parameters in MAST and a P-value below 10^{-4}. Since the N-terminal tails of HCP-3 and its paralogs are highly divergent, we were not able to identify the separase motif efficiently via motif analyses. To identify presence of the

ExxR separase motif, we separately aligned each HCP-3 or HCP-3L protein sequence with *C. elegans* HCP-3 and CPAR-1 (HCP-3L1) either individually or together. This alignment was used to generate the predicted separase motifs shown in Supplementary Figure 3.S5.

3.4.6 Analysis of evolutionary selective pressures

To analyze selective pressures on *CenH3* genes, we compared rates of synonymous (dS) to nonsynonymous (dN) substitutions among *hcp-3* and *hcp-3L* genes. dN and dS between all pairwise combinations of *CenH3* genes were determined using SNAP (Korber 2000) (www.hiv.lanl.gov) on a codon alignment of the histone fold domain (Supplemental Table S1A). dN/dS ratios were used to determine the selective pressures acting on *CenH3* genes.

For all other tests, we generated codon alignments using MUSCLE (Edgar 2004), and manually adjusted them to improve alignments if needed. We also trimmed sequences to remove alignment gaps and segments of the sequence that were unique to only one species. We found no evidence of recombination for any of these alignments using the GARD algorithm at datamonkey.org (Kosakovsky Pond, et al. 2006). We used the alignment to generate a tree using PhyML maximum-likelihood methods with the HKY85 substitution model (Guindon, et al. 2010).

We analyzed selective pressures on *Caenorhabditis hcp-3* and kinetochore proteins using the codeml algorithm from the PAML suite (Yang 1997) (Supplemental Table S1A). We generated codon alignments using MUSCLE (Edgar 2004) via Geneious's Translation Align tool which we manually adjusted if needed to improve alignments. These alignments were used to generate trees using PhyML maximum-likelihood methods with the HKY85 substitution model (Guindon, et al. 2010). To test whether any residues evolve under positive selection, we

compared likelihoods between model 8 (where there are ten classes of codons with dN/dS between 0 and 1, and an eleventh class with dN/dS > 1) and model 7 (which disallows codons with dN/dS > 1) or model 8a (where the eleventh class has dN/dS fixed at 1). To test whether duplicates were evolving under positive of purifying selection, we compared the likelihood of model 0 with dN/dS fixed at 1 (neutral) with that of model 0 with dN/dS estimated from the alignment. In both cases, to determine statistical significance, we performed likelihood-ratio tests between the two models to a χ^2 distribution with the degrees of freedom reflecting the difference in number of parameters between the models being compared (Yang 1997).

3.4.7 C. elegans HCP-3 and CPAR-1 polymorphisms

To determine natural variation in *C. elegans hcp-3* and *cpar-1* genes (Supplementary Figure 3.S6), we used the *Caenorhabditis elegans* Natural Diversity Resource (Cook, et al. 2017). The synonymous mutations in *hcp*-3, as well as the frameshift, synonymous, and non-synonymous mutations in *cpar-1* were identified by the CeNDR variant annotation feature. The *cpar-1* partial deletion was found manually by looking at whole-genome sequencing reads from *C. elegans* strain ECA740 mapped onto the N2 reference genome.

3.4.8 RT-PCR

Total RNA was isolated using TRIzol (Fisher Scientific) from 50-100 L4 or young adult males, females, or hermaphrodites or from a near starved plate of mixed-stage animals. RNA was extracted by chloroform extraction, precipitated using isopropanol, washed with ethanol, and resuspended in 20µl of nuclease-free water. Next, RNA was treated with DNase I (New England Biolabs, 2 units/µl) at 37°C for 60 minutes followed by heat inactivation at 75°C for 10 minutes.

DNase-treated RNA was purified using the RNA Clean and Concentrator-5 kit (Zymo Research) and converted to cDNA using SuperScript III Reverse Transcriptase (Invitrogen) using polydT primers as per manufacturer's recommendations. RNA concentrations used to make cDNA were not kept the same between whole plate, male, and female/hermaphrodite samples except for samples from *C. afra* (in Figure 3.5C), *C. remanei* and *C. sinica*. PCR was done on cDNA using Phusion High-Fidelity DNA Polymerase Kit (New England Biolabs) guidelines according to the manufacturer's recommendations using primers for *hcp-3, hcp-3L,* and *tbb-2*. All primer sequences used are listed in Supplemental Table S2.

3.5 ACKNOWLEDGMENTS

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publish this study. P.R. is a Washington Research Foundation Fellow and H.S.M. is an Investigator of the Howard Hughes Medical Institute.

3.6 FIGURES

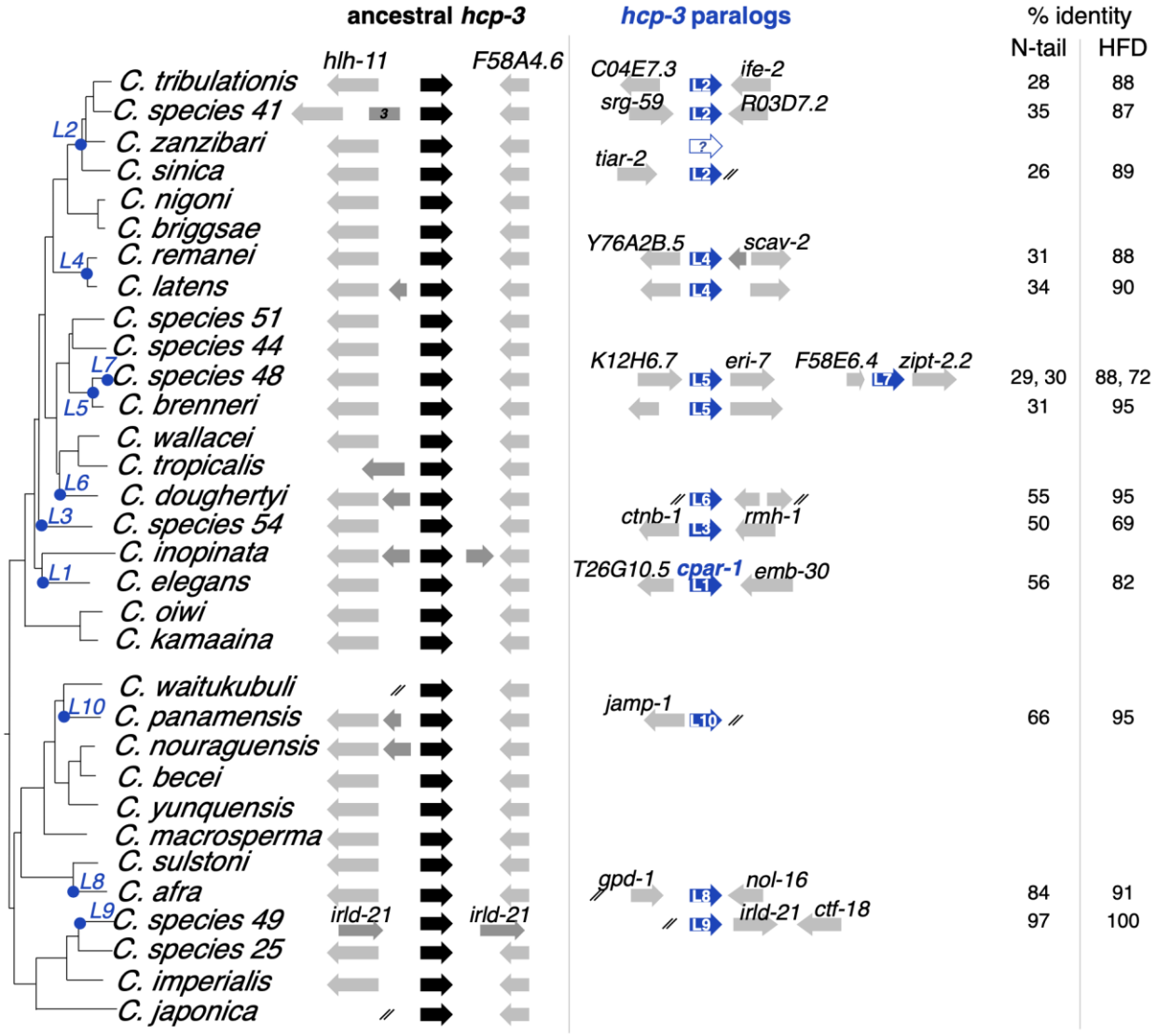


Figure 3.1. Ten independent *hcp-3* duplications in Caenorhabditis species.

A schematic representation of ancestral centromeric histone genes (*hcp-3*, black) and their duplicates (*hcp-3L*, blue) are shown alongside a *Caenorhabditis* species tree (adapted from http://caenorhabditis.org). *hcp-3* duplication events are represented on the species tree with a blue dot and numbered *L1* through *L10*, with paralogs arising from independent duplications assigned different numbers. Genes in the syntenic neighborhood near *hcp-3* and *hcp-3L* are

represented in grey and labelled with their orthologous gene names in *C. elegans*. In some cases, 1-3 genes were inserted between *hlh-11* and *F58A4.6* within the syntenic neighborhood of *hcp-3*. The white arrow with a question mark represents a possible loss of *hcp-3L2* in *C. zanzibari*. Ends of genomic scaffolds are denoted with two slashes. On the right, we show percent amino-acid identities between the paralog and ancestral *hcp-3* of each species (in the N-terminal tail or histone fold domain (HFD)).

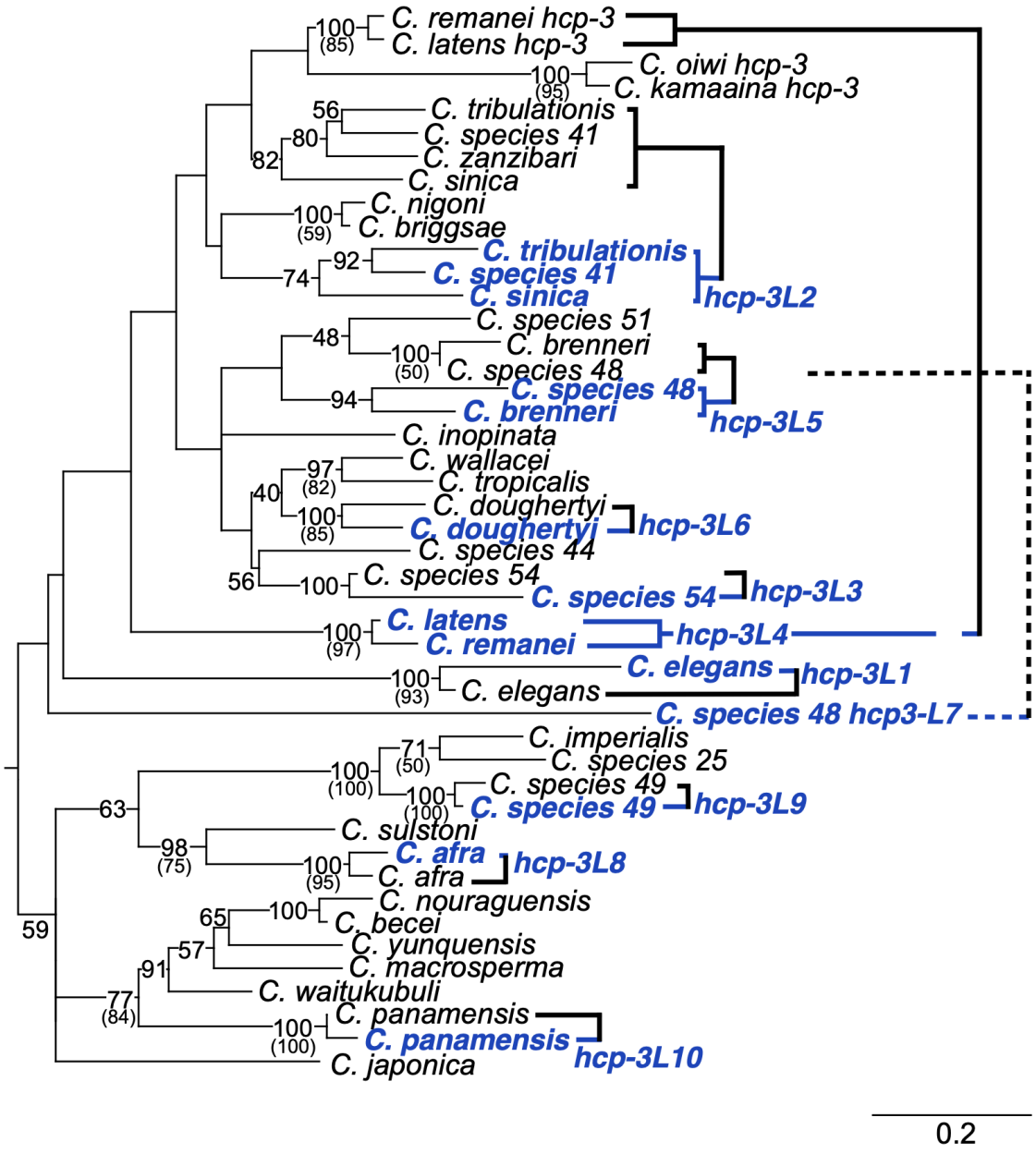


Figure 3.2. Phylogenetic analysis of *hcp-3* and *hcp-3L* genes in *Caenorhabditis* species. A maximum likelihood tree of a DNA, codon-based alignment of the HFD of ancestral *hcp-3* (black) and *hcp-3* paralogs (blue) is shown. Bootstrap values of 40 and above are indicated. Bootstrap values in parentheses are from corresponding nodes from a maximum likelihood tree based on an amino acid alignment of the HFD (see Supplementary Figure S2). In all except a few instances, the nucleotide and amino acid tree are in agreement, with higher bootstrap support

observed in the nucleotide tree. For the exceptions (nodes representing *hcp-3* or *hcp-3L2* in *C. tribulationis*, *C. sinica*, *C. sp41*, and *C. zanzibari*, and the node representing *hcp-3* in *C. nouraguensis*, *C. becei*, and *C. macrosperma*), bootstraps values were not included here since they were lower in the amino acid tree and because they do not alter conclusions from the nucleotide tree. A scale bar (branch lengths, substitutions per site) is shown at the bottom-right. On the right, thick lines show *hcp-3* paralogs from same species, the dashed line shows the second duplicate found in *C. species 48*.

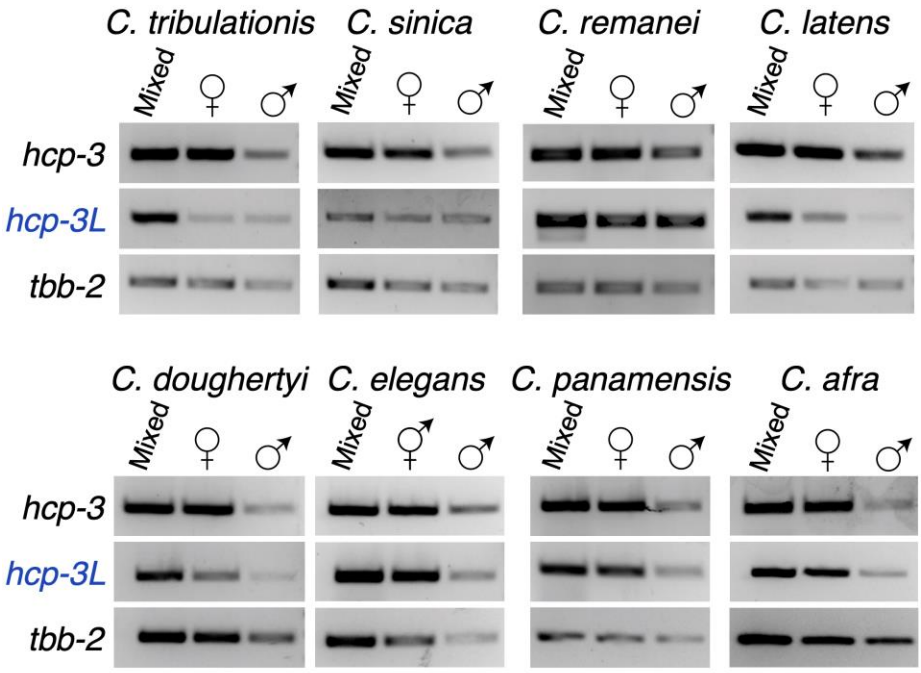


Figure 3.3. *hcp-3L* genes are expressed in both sexes in Caenorhabditis species.

RT-PCR of ancestral *hcp-3* (top), *hcp-3L* (middle), or *tbb-2* (bottom; loading control) in species with *hcp-3* duplicates. RNA from a mixed worm population of various larval stages, L4 or young adult females/hermaphrodites or L4 or young adult males were used.

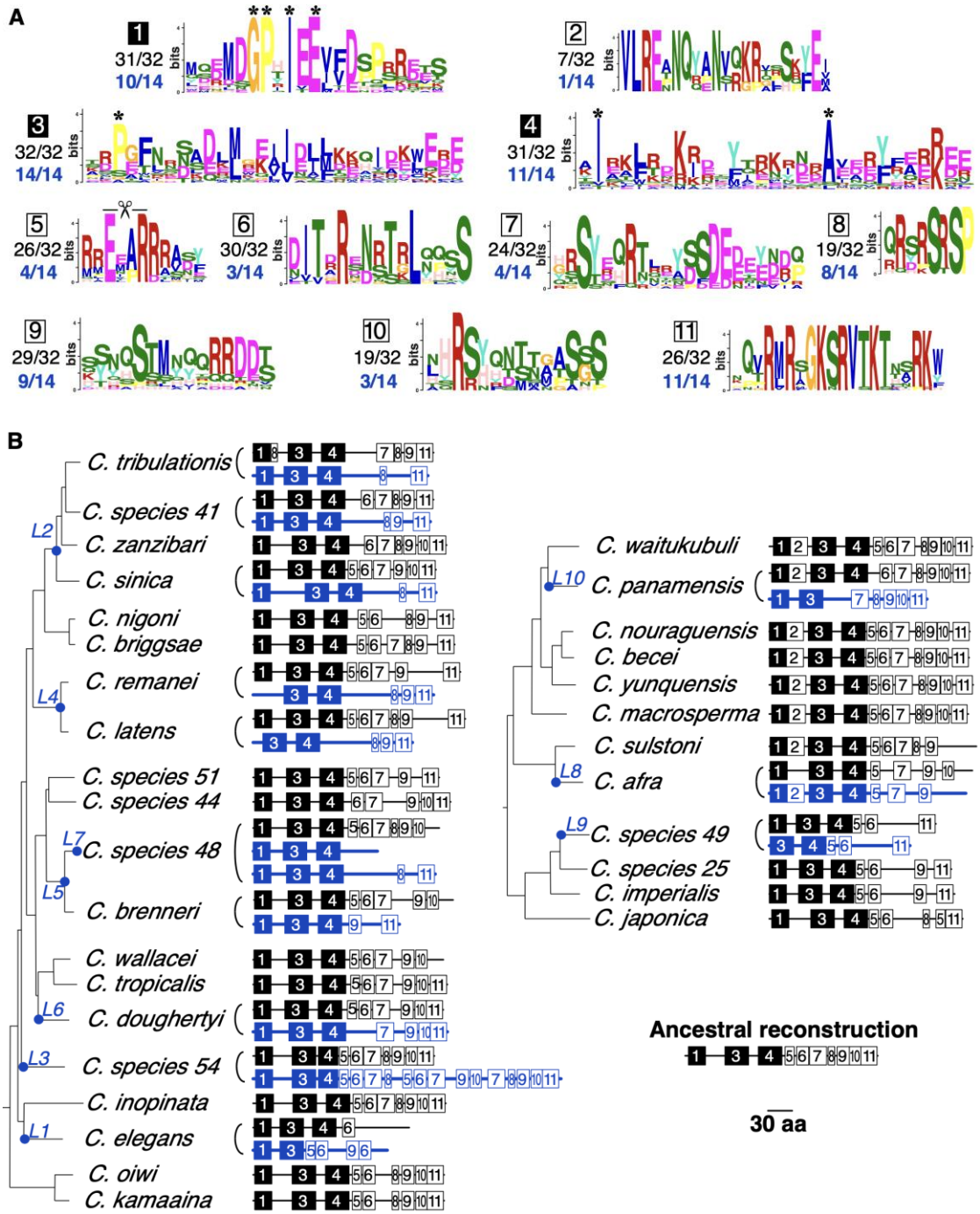


Figure 3.4. Differential retention of N-terminal tail motifs across HCP-3 and HCP-3L proteins encoded by Caenorhabditis species.

(A) Logo plots of eleven protein motifs within HCP-3 N-terminal tails discovered from an analysis of *Caenorhabditis* species without duplications. Motifs 12 and 13 are C-terminal motifs

(not shown, see Supplementary Figure S4) that reside within the HFD. E-values of all motifs were below 10^{-5}. Asterisks above logo plots for motifs 1, 3, and 4 indicate residues that are highly conserved within the motif. Proportion of all 32 ancestral HCP-3 proteins (black) or 14 HCP-3L duplicates (blue) that have retained the motifs are shown. (B) *Caenorhabditis* species tree with schematics of protein motifs that are present (numbered boxes) in ancestral HCP-3 (black) or HCP-3L (blue) in each species is shown. The presence of motif 1 in *C. elegans* and motif 4 in *C. sp54* was not detected by unsupervised MAST searches but was subsequently ascertained through manual alignments (see Supplementary Data File S4). All proteins contained a conserved, C-terminal HFD (not shown). Filled black boxes represent three motifs that show the highest retention in *Caenorhabditis* HCP-3 proteins. A structure of the N-terminal tail of HCP-3 in the last common ancestor of *Caenorhabditis* was inferred based on the retention and loss of motifs in the N-terminal tail. L1-L10 on the species tree indicate *hcp-3* duplication events as in Figure 3.1. A scale bar (number of residues) is shown on the bottom-right.

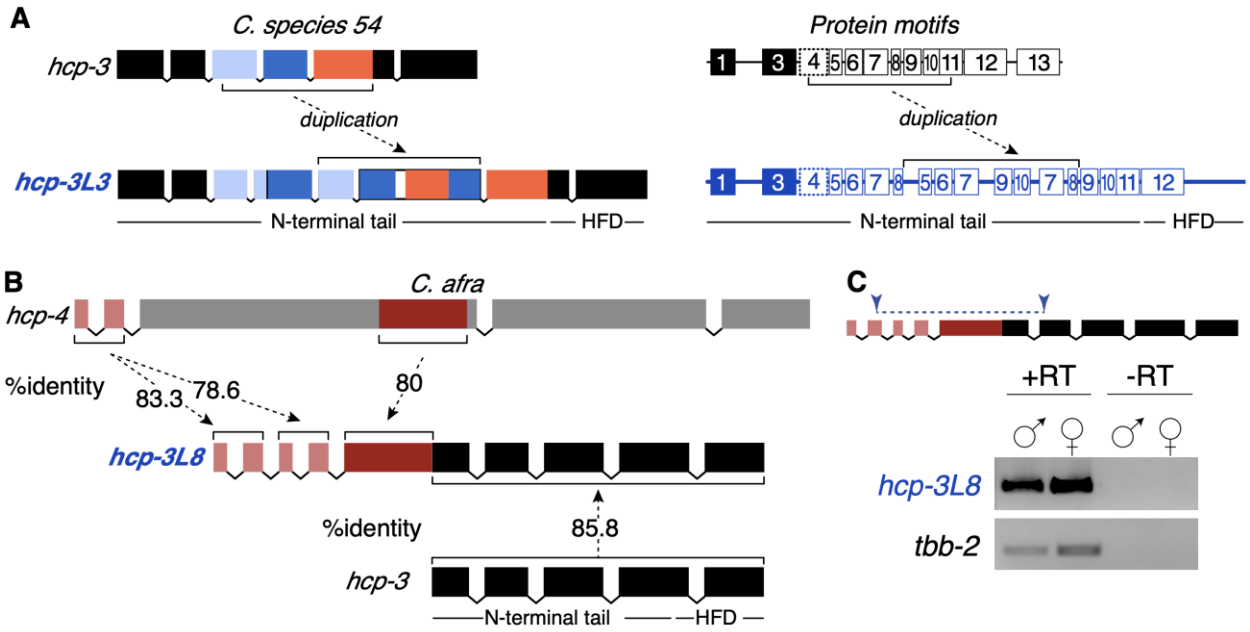


Figure 3.5. Two unusual Caenorhabditis *hcp-3L* paralogs arose by internal duplication or gene fusion.

(A) Schematic of the exon structure (left) and protein motif structure (right) of *C. sp54 hcp-3* (top) and *hcp-3L3* (bottom). Portions of *hcp-3* exon 3 (light blue), exon 4 (dark blue), and exon 5 (orange) are duplicated within the N-terminal tail of *hcp-3L3* (dashed arrow). Similarly, motifs 5-10 are duplicated within the N-terminal tail of HCP-3L3. Motif 13 resides within the HFD and is missing in HCP-3L3. The HFD is not within the duplicated region. (B) Schematic of the exon structure of *C. afra hcp-3L8* (middle) with homology to *C. afra hcp-4* (top) and *C. afra hcp-3* (bottom). The first five exons of *hcp-3L8* are homologous to *C. afra hcp-4* exons 1 and 2 (light red) as well as a portion of exon 3 (dark red). The last five exons of *hcp-3L8* are homologous to *C. afra hcp-3* (black). The HFD and the N-terminal tail of *hcp-3* are denoted. Percent amino acid identity between protein-coding exons are shown. (C) Primers designed to span exons that are homologous to *hcp-3* and *hcp-4* within *hcp-3L8* (top). Schematic of the gene shows primers used to amplify the *hcp-4-hcp-3* fusion region (top, blue) in RT-PCR of *C. afra hcp-3L8* and *tbb-2* in

males and females (bottom) to confirm expression of a chimeric transcript. +RT and -RT indicate cDNA preparation with or without reverse transcriptase enzyme, respectively.

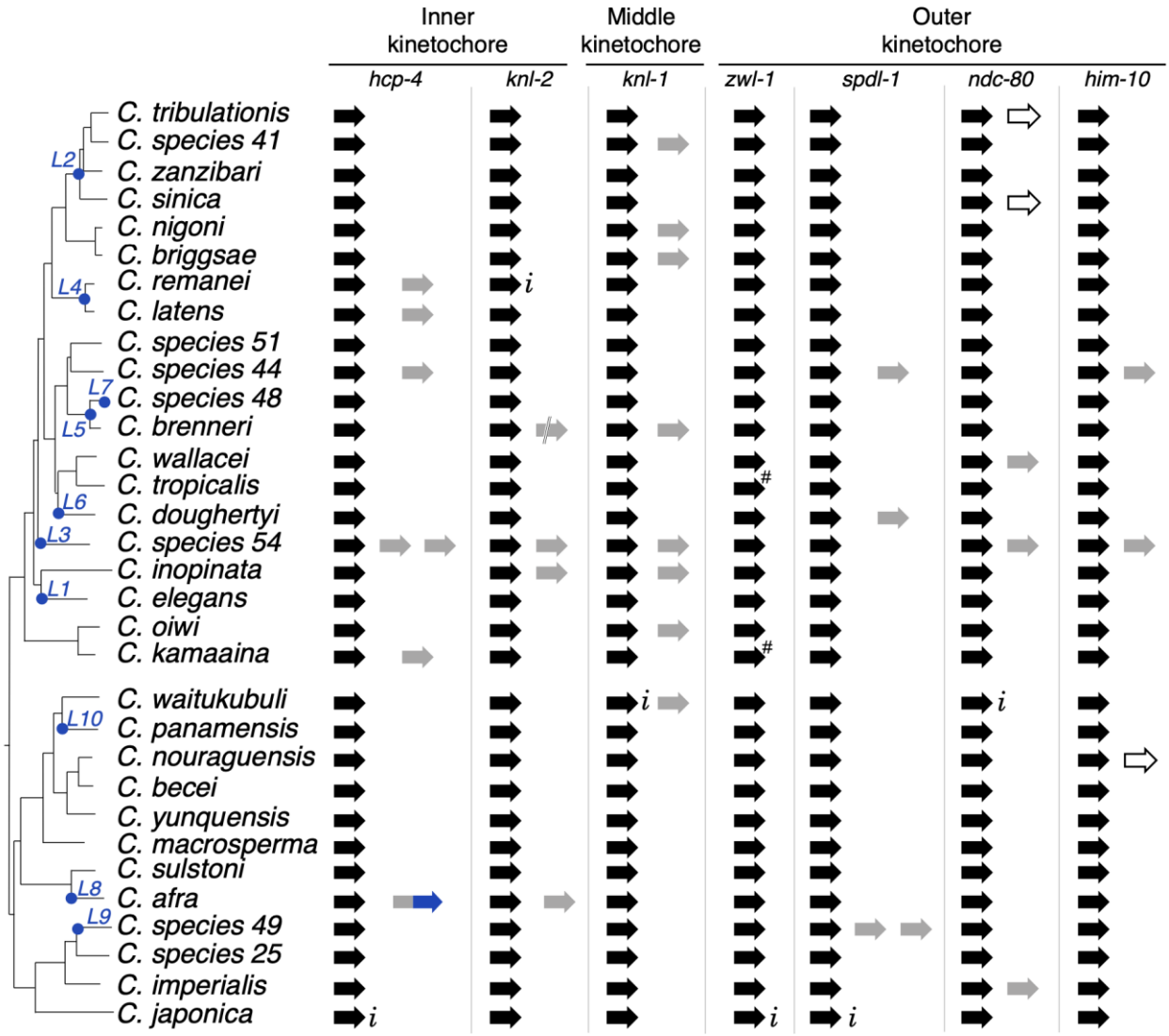
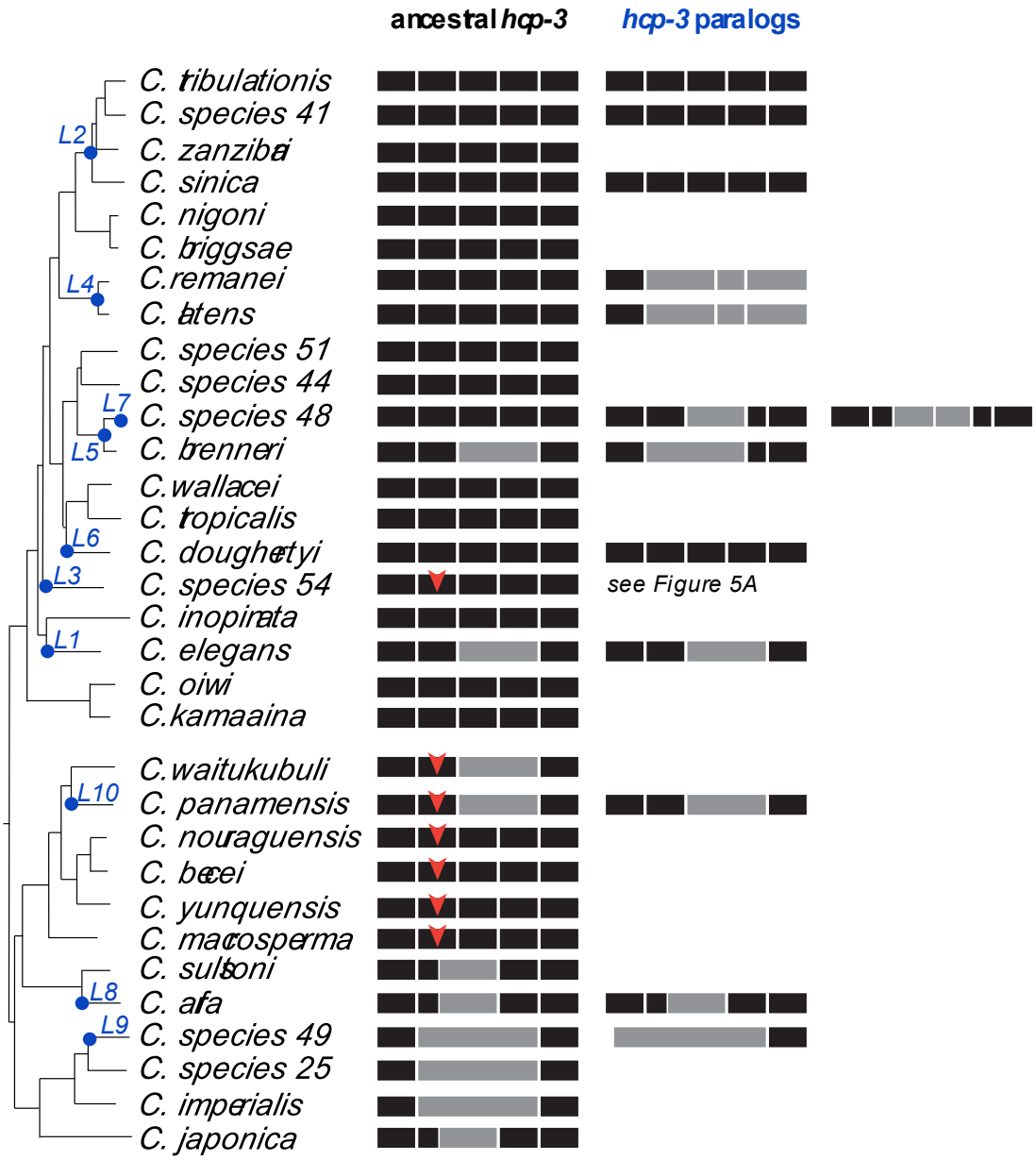


Figure 3.6. Duplication of kinetochore proteins in Caenorhabditis species.

A schematic representation of ancestral (black) and duplicate (grey) copies of seven kinetochore genes (*hcp-4*, *knl-2*, *knl-1*, *zwl-1*, *spdl-1*, *ndc-80* and *him-10*) shown alongside a *Caenorhabditis* species tree. *hcp-3* duplication events are denoted as a blue dot on the species tree, as in Figure 1. The unique fusion between *C. afra hcp-4* and *hcp-3* duplicates is shown in grey and blue. Incomplete sequence information in genomic scaffolds is denoted with *i* and apparent pseudogenes are denoted as unfilled arrows. Double slash in *C. brenneri knl-2* duplicate indicates

the sequence was split between two scaffolds. # indicates two potential pseudogenization events in *zwl-1* that are likely to represent sequencing errors.

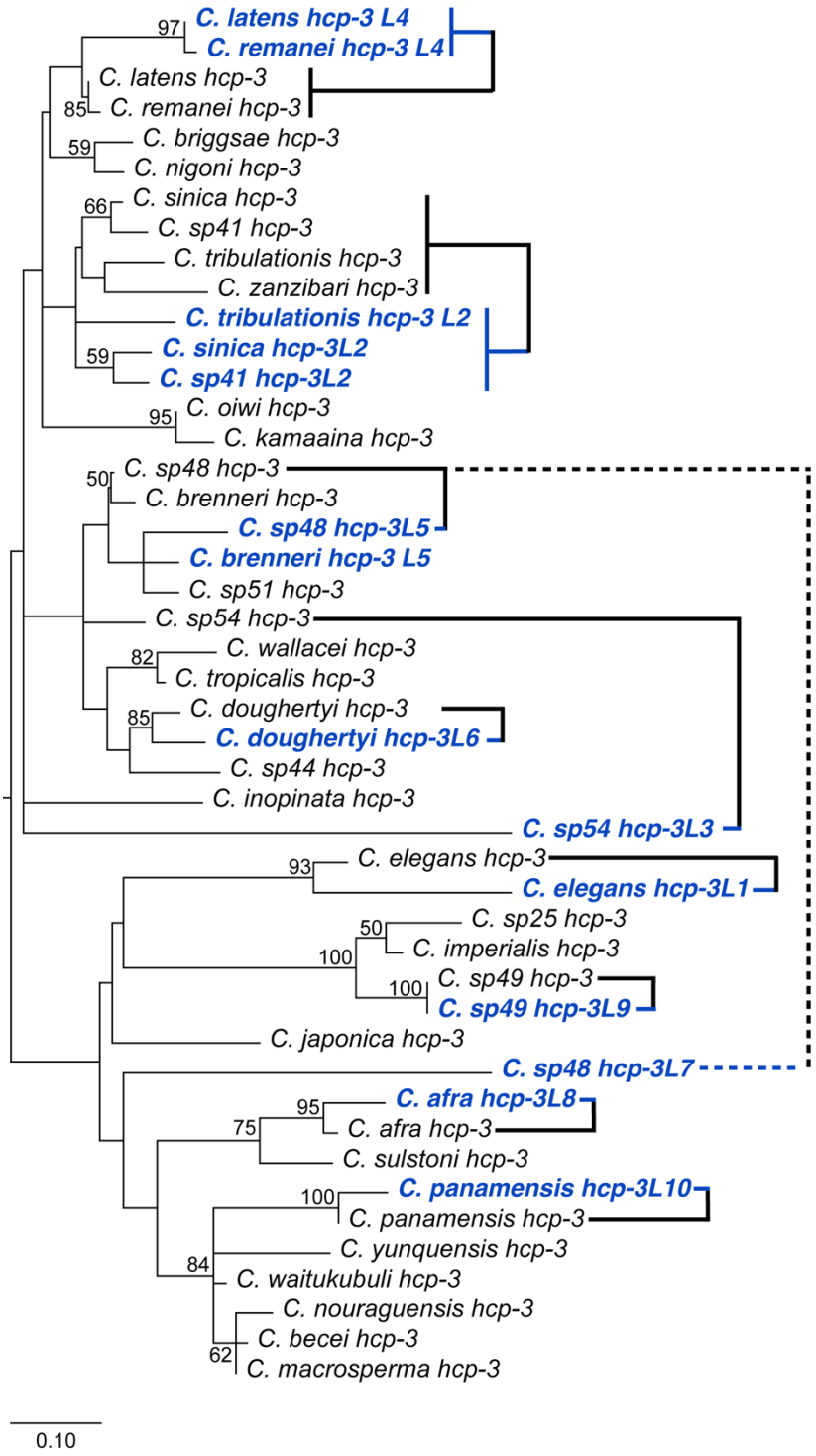
3.7 SUPPLEMENTARY MATERIALS



Supplementary Figure 3.S1. Exon-intron junctions are largely retained in *Caenorhabditis hcp-3* and *hcp-3L* genes except for intron gains and losses that are likely a result of partial retrotransposition.

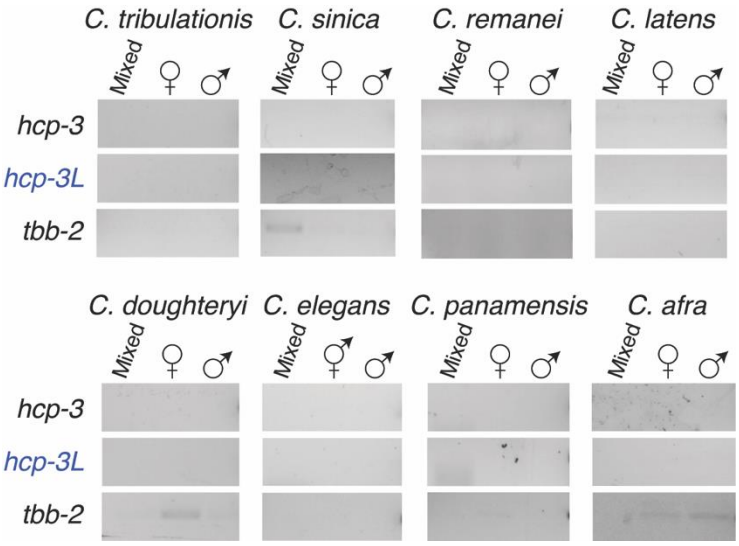
Schematic of the exons of *hcp-3* and *hcp-3L* genes in *Caenorhabditis* species. Each box represents an exon with black boxes showing ancestral exons. Grey boxes depict exon fusion

events that are a likely consequence of partial retrotransposition and overwriting of the genomic locus as has been previously observed (Robertson 1998; Cho, et al. 2004; Kiontke, et al. 2004). Red arrows indicate insertion events that likely create new introns. Exon duplications for *C. sp54 hcp-3* and *hcp-3L3* are discussed in more detail in Figure 5A in the main text. An N-terminal extension to *C. afra hcp-3L8* (homologous to *hcp-4*) is not shown but discussed in more detail in Figure 3.5B.



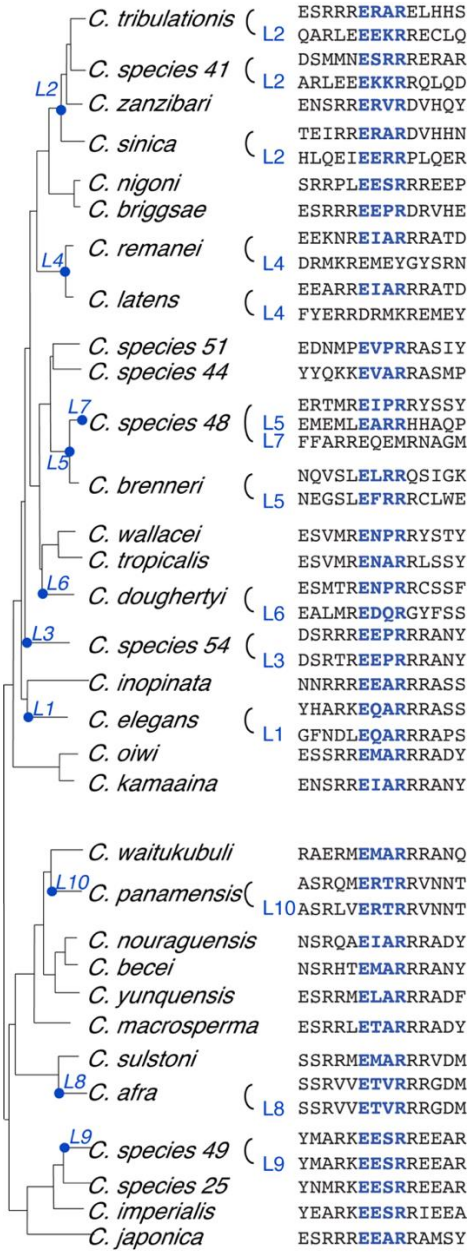
Supplementary Figure 3.S2. Maximum-likelihood phylogenetic tree based on an amino acid alignment of HCP-3 and HCP-3L proteins encoded by Caenorhabditis species.

A maximum likelihood tree based on an amino acid alignment of the histone fold domain (HFD) of ancestral *hcp-3* (black) and *hcp-3* paralogs (blue) is shown as a phylogram (branch lengths are scaled to evolutionary divergence indicated). Bootstrap values of 40 and above are indicated. Overall, this phylogeny is much more poorly resolved than one based on the nucleotide alignment (Figure 2) and does not fully recapitulate known relationships between *Caenorhabditis* species or relationships between *hcp-3* and *hcp-3L* genes from the same species. However, well-resolved nodes agree between both the protein and nucleotide phylogenies and are also indicated in Figure 2.



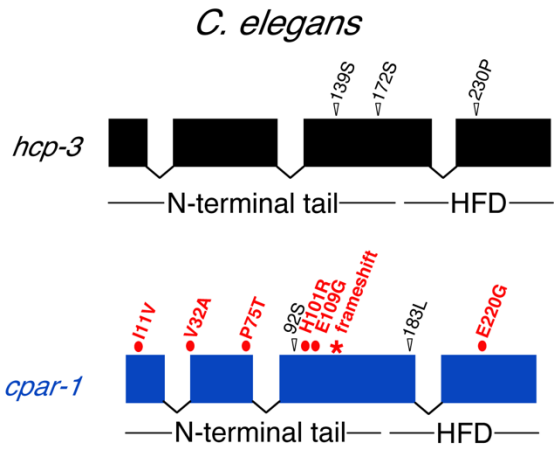
Supplementary Figure 3.S3. RT-PCR controls for expression analysis of *hcp-3* and *hcp-3L* genes. No reverse transcriptase (-RT) PCR control of ancestral *hcp-3* (top), *hcp-3L* (middle) paralogs, or *tbb-2* (bottom) loading control genes in selected species with *hcp-3L* duplicates. RNA from a mixed worm population of various larval stages, L4 or young adult females/hermaphrodites or L4 or young adult males were used. In some cases, we see very faint bands in the *tbb-2* -RT controls compared to +RT samples.

Supplementary Figure 3.S4. Conserved motifs identified in the HCP-3 histone fold domains. Logo plots of conserved motifs 12 and 13, which reside in the HCP-3 histone fold domain. Proportion of all 32 ancestral HCP-3 proteins (black) or 14 HCP-3L duplicates (blue) that have retained the motifs are shown. All HCP-3 and HCP-3L proteins contain motifs 12 and 13, except for *C. sp54* HCP-3L3 which has a poor statistical match to motif 13 owing to high divergence (see Figure 5A).



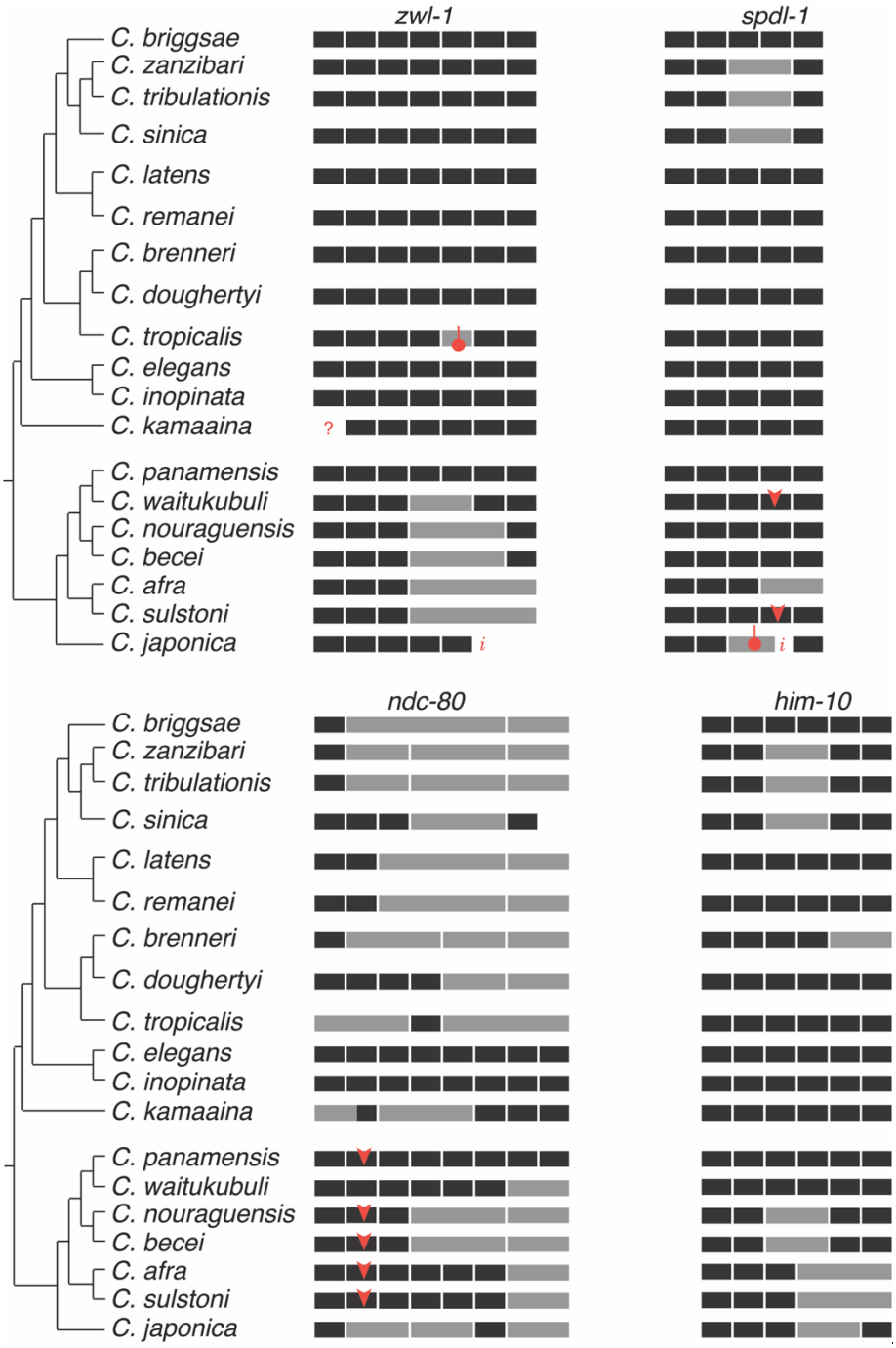
Supplementary Figure 3.S5. The ExxR putative separase cleavage site is retained in most Caenorhabditis HCP-3 and HCP-3L proteins.

Alignments of ExxR residues (blue colored) and flanking residues in HCP-3 and HCP-3L proteins are shown alongside a *Caenorhabditis* species tree.



Supplementary Figure 3.S6. A higher frequency of function-altering mutations is observed in *cpar-1* compared to *hcp-3* within natural C. elegans populations.

Schematic of exon-intron structure of *hcp-3* (top, black) and *cpar-1* (bottom, blue) coding regions in *C.* elegans, indicating the N-terminal domain and HFD. Natural variation found in *C. elegans* strains is indicated by arrowheads (black) and ovals (red) that represent synonymous and nonsynonymous mutations respectively. Three synonymous mutations and zero nonsynonymous mutations were found in *hcp-3*, whereas six nonsynonymous and two synonymous mutations were found in *cpar-1*. In addition, a single nucleotide insertion in *cpar-1* that causes a frameshift resulting in an early stop codon was found.



Supplementary Figure 3.S7. Other kinetochore genes also display patterns of partial intron loss in Caenorhabditis species.

Schematic of the exon arrangements of four kinetochore proteins (*zwl-1*, *spdl-1*, *ndc-80* and *him-10*) in a representative set of *Caenorhabditis* species. Each box represents an exon with black

boxes showing ancestral exons and grey boxes showing fusion events between exons that likely arose due to partial retrotransposition and overwriting of the genomic locus (Robertson 1998; Cho, et al. 2004; Kiontke, et al. 2004). Red arrows indicate insertion events that likely create new introns and red dots represent deletions in exons. Incomplete genomic sequence information is indicated with an *i.* Although we were unable to identify exon 1 of *C. kamaaina zwl-1* using homology, it is unlikely to have been pseudogenized, since *zwl-1* is an essential gene and is present as an intact gene in all related species.

Supplementary Data 3.S1: Pairwise alignments of Caenorhabditis HCP-3 HFD and N-tail used for Figure 1

***C. tribulationis* HCP-3/HCP-3L2 N-tail alignment**

>C_tribulationis_HCP-3_N-tail
---MYA--
HTGPIIEEVEEAATDGQTVHRDWSKDPDVLRIRRELQKLMILPGFNNNADLMQRAITILV
EQVDEWKLDQDIDGWNICRQEKIESFLPRIANFKNKRAQAIDQFYKERDSMINESRRRER
ARELHHSNDFGISGRELDHSSRLHQLSRRDSCASRVERYHSSDEDEENHPVPRYRSRSPG
PSSSYNQST-MRQRRDDVPQPV-RMRSGKSRVTKTRNAKWRPG
> C_tribulationis_HCP-3L2_N-tail
MEQMYENQHT-PIIEELFDCSS---VVERE------
VERVKHEIQALTSQSDFNKNYASMKEVINILTRQIAAWDADEDMGGSHPIRLRSIEKFHA
KRVLFTEKLEAAERAYYEKKQARLEEEKRRE---CLQDRGKIAGQNNQLCHR-
QGHRYERDDSSDDSSDEENQRQRSRACSPQRRNHPSTSSQQYRVNRHVDYHKKHQNVS
QKQQRLRAGINAVTKTKVRKFRPG

***C. tribulationis* HCP-3/HCP-3L2 HFD alignment**

>C_tribulationis_HCP-3_HFD
QKALREIRKYQKSTDMLIQKAPFARLVHEIIQETTLFSHDFRIRADALMALQEASEAFMV
EMFEGSFLICNHAKRVTLMPTDIQLYRRLCLR
>C_tribulationis_HCP-3L2_HFD
QKALAEIRRYQKSTDMLIQKAPFARLVHEIIQESTTLSRDFRIRSDALMALQEGAEAFMV
EMFEGSALICNHAKRVTLMPTDVQLYRRLCLR

***C. sp41* HCP-3/HCP-3L2 N-tail alignment**

>C_sp41_HCP-3_N-tail
---MYA-
HTGPIIEEVEDGPAEGHTVHRDWRQDTDVQRLGREIQKFISLPDFSKNADLMQRAIHVLE
KQVDEWKLNQELDGWDHHRQQKIELFQPKIAKFKEKREEAINHYYDVKDSMMNESRR
RERAREVLHNTDQNITGFGNSTRLYPNSRRQSFAPRKERYQSSDE--
DEENDPVPRRRSRSPGPSSSYSHSAMYQLRDD----------
SNAPQQQRMRSGKSRVTKTKNRKWRPG
>C_sp41_HCP-3L2_N-tail
MDQLYENHHTPVIEEILD---TEFVVERE------
VERVKHDIQLITSQPDFNKNYDSMKEVIDILARQILDWEADEEMSGSHPMRRRIIDKFQA
KKVLFTEKLEAAERAYYERKRARLEEEKKRRQLQDC-----GNIGGEQNR-----
HARFQFMGHRNGRDDSSDDSSDGENRQVQRRRSRSRSPQHR-
NHSSQQHRRDDRHADGHRNYQSTSKNQPRLRAGINGVTKTKVRKYRPG

***C. sp41* HCP-3/HCP-3L2 HFD alignment**

>C_sp41_HCP-3_HFD
QKALSEIRKYQKSTDMLIQKAPFVRLVNEIIQEATSFSKEFRIRADALMALQEASEAFMVE
MFEGSVLICNHAKRVTLMPTDIQLYRRLCLR
>C_sp41_HCP-3L2_HFD

QKALAEIRQYQKSTDMLIQKAPFARLVHEIIQDSTNFSRDFRIRADALMALQEAAEAFMV
EMFEGSTLICNHAKRVTLMPTDLQLYRRLCLR

***C. sinica* HCP-3/HCP-3L2 N-tail alignment**

>C_sinica_HCP-3_N-tail

MYGHTGPLIQEIDETPTEGSTAVRDWTTDEDVRRLGKEVQKLTCQEGFTKNANLMRRLI
ELLEKQVDEWKEDQDIHGHDHCRQQKIQCFEKRIADYKKKCERSIRRYCDQRDSRTEIR
RERARDVHHNSNYDITDRGDSIRLNQHYHRQSLAPREESYHSSDDDEENIPPRLYRSGRS
TMYQQRQDDSNVHYRSHHSTLGASSSQQVRMRSGKSRVTKTHNRKWRPG
>C_sinica_HCP-3L2_N-tail
MDHVPPPLTHQPPSLIAAFLPRILVFEVKMQAMQCTPIIEEIHEAPT---------
ALEMEIEVVKHNIKKLTSQPDFSKSYSLMEGAITILKKQIERWEYAERRDGPDGARQEHL
AKFRLKMEQFEEKLKAAERAYYEKKKAHLQEIEER-RPLQERQN----------
AINRQSFEQRRRERRNSSDESSDDETE---QFHHRSRSRSPRRNQLTTSNLQRHRQPT-------
QPRLRQGVDRISKTKARKWRPG

***C. sinica* HCP-3/HCP-3L2 HFD alignment**

>C_sinica_HCP-3_HFD
QKALAEIRKYQKSTDMLIQKAPFARLVHEIIQEATSFSKEYRIRADALMALQEASEAFMV
EMFEGSVLICNHAKRVTLMPTDIQLYRRLCLR
>C_sinica_HCP-3L2_N-tail
QKALAEIRQYQRTTEMLIQKAPFARLVHEIIQDATSFSRDFRIRADALMALQEAAEAFMV
EMFEGSVLICNHAKRVTLMPTDLQLYRRLCLR

***C. remanei* HCP-3/HCP-3L4 N-tail alignment**

>C_remanei_HCP-3_N-tail
MYQMHHNGPRIEEMVDPPSRST-
TNQLKNDTEYIKSEYRRISHLPDFNRDPELIQEVMNLTKRYIEKWLREERD-
EPNMERQGWIERFKTKLREWETKKETAEDEYYTRRDASSNEEKNREIARRRATDSQMNI
TGLHDSTRLNQQSYSRSYENRNRRYSSDEDDDENMAPQRRQRSRSPPSFAHHQRRDDTG
SYYRSHHTQNSSNQRTHNTDFSSHYRGQYGPSTSQNVGMPS--
NAQNVRMRSGKSRVTKTRSRKWRPG
>C_remanei_HCP-3L4_N-tail
---MAPLLIAVSSFVVCSAILIYLCSKKPKTIDLESEI-
GITSRKNFNRDSDSMQEVIDIMTRQINKWEQLEDDYGPDATRQRNIEAFQRKRDLWEEK
KEQAERAFYERRD-----RMKREM----------EYGYSRNQIRRERRMDDDSDSD-
VMEDDRRQ----PLGNLDYSRRDNYVRLREAKPMALLNRDRSRSRSPLLLPR-
QDHPSTSLQLRRPNIPSTPPVRVRPGKSRVTKSKNRKWRPG

***C. remanei* HCP-3/HCP-3L4 HFD alignment**

>C_remanei_HCP-3_HFD
QRALEEIRKYQKSTDMLIQKAPFARLVHEIMREATSESQDFRIRADALMALQEAAEAFM
VEMFEGSVLICNHAKRVTLMPTDIQLYRRLCLR
>C_remanei_HCP-3L4_HFD

QKALLEIRKYQKSTDMLIQKAPFARLVQEILRETTNESHDYRIRADALMALQEGAEAFM
VEMFEGSVLISNHAKRVTLMPTDVQLYRRLCLR

***C. latens* HCP-3/HCP-3L4 N-tail alignment**

>C_latens_HCP-3_N-tail
MYQVYHNGPRIEEMVDPPPKSTTNQLKIDTEYIKSEYGRISSHSDFNRNPDAIQEVIDLAR
RYIEKWQREERD-
EPNMDRRGWIERFKTKLREWETKKETAVDDYVTRRNASSNEEARREIARRRATDSQLNI
TGLQDSTRLNQQSYSRSYENRNRRYSSDEDDDENMAPQRRQRSRSPSSFAYHQNTLNHQ
RRDDTGSYYRSHHTQNSSHQRTHNTDISSHYRRQNGPSTSQNVVMPSNTQNVRMRSGK
SRVTKTRNRKWRPG
>C_latens_HCP-3L4_N-tail

MDQIKEEIEAITSRKNFNRDSDAMQEVIDIMTRQINKWEQLEDDYGPDATRQRNIEAFQR
KRDSWEEKKEQAERAFYERRD-----RMKREMEY----------
DNGYSRSQVRRERRMDDDSDSDV-MEDDGRQ-
PLGNLDYSKRNNFVRGREAMPMKVVNRDRSRSRSPLPRPHHPSTSLQLRRPNIPSSP-----
P-----VRVRPGKSRVTKSKNRKWRPG

***C. latens* HCP-3/HCP-3L4 HFD alignment**

>C_latens_HCP-3_HFD
QRALEEIRKYQKSTDMLIQKAPFARLVHEIMREATSESHDFRIRADALMALQEAAEAFM
VEMFEGSVLICNHAKRVTLMPTDIQLYRRLCLR
>C_latens_HCP-3L4_HFD
QKALLEIRKYQKSTDMLIQKAPFARLVQEILRETTNESHDYRIRADALMALQEGAEAFM
VEMFEGSVLISNHAKRVTLMPTDIQLYRRLCLR

***C. sp48* HCP-3/HCP-3L5 N-tail alignment**

>C_sp48_HCP-3_N-tail
MFRVTDGPTIEEVVETQLTEDTAEAEVRRDYDTILEELRAVLGVPGANRDQERLGRGLCI
LEKGIDKFEEDEDN-
QPLEIRRQYLGKLREKYSSCEAKLREAENAFHERKEREYEERTMREIPRRYSSYRDTDIT
RRNNTTGLYHHSQQSSSNYRQQGYSSDEEMENFPSSHRDRYRSPPRKFSHSTMLQQRRD
ISPVVNRSHQQSSASSQQVRMRSGKSRVTKTTRKYRPG
>C_sp48_HCP-3L5_N-tail
-MMDEDSPRIEEIVE----
EDEAEKDVKKEFDEYRREIEAVTSLPGFNRDSGKMTQVLRIMEKAIGKWEEDEENLGSI
QCRRRCLLEFKRRHDNYEKIIEKAEDDFYKRREMEMLE----------ARR-------
HHAQPESRQYPDGG----------AEQRQTIP----------
KMRAGKSSVTKKPKKFRPG

***C. sp48* HCP-3/HCP-3L5 HFD alignment**

>C_sp48_HCP-3_HFD
QKALAEIRQYQKSTDLLIQKAPFARLVHEIIREATSNSGDYRVRADALLALQEGAEAFMV
EMFEGSVLICNHAKRVTLMPTDIQLYRRLCLR

>C_sp48_HCP-3L5_HFD
EKALAEIRQYQRSTDLLIQKAPFARLVHEIVSEATSSSGDYRIRADALMALQEGAEAFMV
EMFEGSALICNHAKRVTLMASDVQLYRRLCLR

C. sp48 **HCP-3/HCP-3L7 N-tail alignment**
>C_sp48_HCP-3_N-tail
MFRVTDGPTIEEVVETQLTEDTAEAEVRRDYDTILEELRAVLGVPGANRDQERLGRGLCI
LEKGIDKFEEDEDNQ-PLEIRRQYLGKLREKYSSCEAKLREAENAFHERKEREYEE---
RTMREIPRRYSSYRDTDITRRNNTTGLYHHSQQSSSNYRQQGYSSDEEMENFPSSHRDRY
RSPPRKFSHSTMLQQRRDISPVVNRSHQQSSASSQQVRMRSGKSRVT-KTTRKYRPG
>C_sp48_HCP-3L7_N-tail
MLEDHDSPHIEELVDAD-
DEKNAEEAVKKEFFEFKNEIEKISCLPNFTKDSEKLRQVLAVIGKAIDKWEEDEDNEGSIE
VRRKYLKELKERYFKYDRIIEKAEQKFFARREQEMRNAGMKTTRYYPGHGSSNHQYPFS
REEDVV----NSRSPSRHRPNSGHPHQHAPKLSSCRQQHGYNYWDEKLCSRS---
PRRSNLPIRDQFHQ—SSTNAQRELKAGKSRVTKKVSHKYCSG

C. sp48 **HCP-3/HCP-3L7 HFD alignment**
>C_sp48_HCP-3_HFD
QKALAEIRQYQKSTDLLIQKAPFARLVHEIIREATSNSGDYRVRADALLALQEGAEAFMV
EMFEGSVLICNHAKRVTLMPTDIQLYRRLCLR
>C_sp48_HCP-3L7_HFD
QRAIAEIKHYQKTTELLIQKAPFARLVQEVVQEATSESSSYSIRTDALSALQEGAEAFLVE
MFEGSSMIANHAKRATLGSTDLKLYRRLCLR

C. brenneri **HCP-3/HCP-3L5 N-tail alignment**
>C_brenneri_HCP-3_N-tail
MFHLSDGPTIEELVDTQQLENTAEAEFKEELDVIKKELAAVLAIPDIHRNREALEKSIRILE
KAIDKWEEDEENQVSLELRRQSIGKFKEQRRSCKQKLRDAENAFHERREREYEERTMRE
IPRRYSSFRDTDITRRNNTTGLYHHSQQSSSNFRMQEYSSDEEIENIPSSHRDRYRLEKCLII
VFQNLYFSYPPKKISHSTMLQQRRDISPVVYRSQQQSSAGSQQERMRSGKSRVTKTT--
RKHRPG
>C_brenneri_HCP-3L5_N-tail
MMMMEDSPHIEEIVEVEE----
AEIEVKRQFEEYKKEIEEVARLPGFNKDSGKMNQVIRIMDKAIEKWEEDEENEGSLEFRR
RCLWEFKHRHNNYKKIIEKAEEDFYKRRE---EAMKMVSILNRTPQSHSYSQTR-
DGTAGSYR-TQATSSGPRHRAFESDEE--------RD----------
QRERMQVGRSTISKKTPKRKYRPG

C. brenneri **HCP-3/HCP-3L5 HFD alignment**
>C_brenneri_HCP-3_HFD
QKALAEIRKYQKSTDLLIQKAPFARLVHEIIREATTNSGDYRVRADALLALQEGAEAFMV
EMFEGSVLICNHAKRVTLMPTDIQLYRRLCLR
>C_brenneri_HCP-3L5_HFD
QKALAEIRKYQKSTDLLIQRAPFARLVHEIVREATASSGDYRVRADALMALQEGAEAFM
VEMFEGSVLICNHAKRVTLMPTDIQLYRRLCLR

***C. doughertyi* HCP-3/HCP-3L6 N-tail alignment**

>C_doughertyi_HCP-3_N-tail

---MYDDSDRPTIEEIDDTENSGYR-TAEAIFQERAEEVSKQIKTLLKNPTT---
DDLKQVIRIMSRSIDEWAEEEDNEGSIRARTTAIKTLTRKRNDYEMSLARKENEFLRKRG
QRHEEESMTRENPRRCSSFRDTDITRRTDRTGLNQSSYQGPSSNQQTHYYSSDEDYENRP
RSNRDPYRYNDSPQRSHQSSMYQQRRDASPPLHRSHTTLNGTSGSQQVRMRSGKSRVT
KTTARKYRPG

>C_doughertyi_HCP-3L6_N-tail

MMQMYDDG—
PTIEEILDNHHSGGPITAETIFKEDFDDVSKQIKTLTRDGSTKNADNLKQIISIMSRSIDKWA
EDEDMEGSIRMRKDAIEAFTKKRNEFREKITHAEDEYMRRKRQRMDEEALMREDQRGY
FSSRDNDMARHAGGTALDFNYQQGTSSNYRARYYSSDEDYENAPRSDRDRYRYPDSPQ
KSNQLATYHQRRDVSYSPSRSHQPMNGASSSTQVRMRAGKSRVTKKNSRKFRPG

***C. doughertyi* HCP-3/HCP-3L6 HFD alignment**

>C_doughertyi_HCP-3_HFD

QKALAEIRQYQKSTDLLIQKAPFARLVHEIIREESSQTDFRVRADALLALQEAAEAFMVE
MFEGSVLICNHAKRVTLMPADIQLYRRLCLR

>C_doughertyi_HCP-3L6_HFD

QKALAEIRQYQKSTDLLIQKAPFARLVHEIIREETAVDDFRVRADALLALQEAAEAFMVE
MFEGSVLICNHAKRVTLMPADVQLYRRLCLR

***C. sp54* HCP-3/HCP-3L3 N-tail alignment**

>C_sp54_HCP-3_N-tail

MEQIYDDMRGRIEEIVDPPSRNTTVFRDLTQDQDSDVDRIRNQMKLIMQKPNFNTSIHEM
QKVISILDDQINKWESEEELRDNLFEKMERAKADYYKRKEAEDDSRTREEPRRRANYTD
MDITDRDNATRLNHL----------
SYQRSYSHRNQIDNSDDDDIENMT----------
-----RSRRDRSRSPPSHSYYQSTMNQQRRDESYAHQRSHYSMSVPSSSHQVRMRSGKSR-
VTKTNSRKWRPG

>C_sp54_HCP-3L3_N-tail

MRQIYDDMRERIEEIVDPPSRNSTVFRDLTQDQDSDLERVRNQIKRIIEKPGSNTSIHELQK
VINILDDQIYKWESEEETRDNYLEKMERAKAEYYKRKEAQDDSRRREEPRRRANYTDM
DITDRYNATRLNHLSSYQRSYSHQNQIDNSDDDDIENMIPRSRRDRSWSPPSHYKRKVAQ
DDSRTREEPRRRANYTDMDITDRGNSTRLNHLSSYQRSYSHRNQIDNSDDDDIENMTRK
YNTIFKSPPSHGYHQSTMNQQRRDESYAHERSHYSKSAPSNSHQVRMSSYQRSYSHRNQ
SDNSDDDDIENMIPRSRCDRSRSPPSHGFHQSTMNQQRRDKSYAHQRSHYSMRAPSGSH
QERMRSSKSRRVTKKNYRKWRPG

***C. sp54* HCP-3/HCP-3L3 HFD alignment**

>C_sp54_HCP-3_HFD

HKALSEIRMYQKSTDLLIQKAPFARLVHEIIRDTTSNSQDYRVRADALLALQEAAEAFLV
EMFEGSVLICNHAKRVTLMPTDIQLYRRLCLR

>C_sp54_HCP-3L3_HFD

QKALYEIQKYQKSTDLLIPKAPFARIVHEMIHKATSTSHDLRVRANTFLPLQEAAEAFLVQ
MFHGSMKYCNSAKRVTLMQTDIQNYRSP---

***C. elegans* HCP-3/HCP-3L1 (CPAR-1) N-tail alignment**

>C_elegans_HCP-3_N-tail
MADDTPIIEEIAEQNESVTRIMQRLKHDMQRVTSVPGFNTSAAGVNDLIDILNQYKKELE
DDAANDYTEAHIHKIRLVTGKRNQYVLKLKQAEDEYHARKEQARRRASSMDFTVGRN
STNLVDYSHGRHHMPSYRRHDSSDEE-----NYSMDGTN-
GDGNRAGPSNPDRGNRTGPSSSDRVRMRAGRNRVTKTRRYRPG
>C_elegans_HCP-3L1_CPAR-1_N-tail
MADDGPIIEEIAEKNGRVARIMQRLQHDTQRVTSVPGFNTSATGYADLIALLDQYKNDLE
AVGFNDL----------
EQARRRAPSVDITVGSNSTNLVDYSHGRHDMPSHRRHDSSDEEITAANSHHQSPINVGNR
NDTDGTNGRNGSRAGSSSSDRVRMIAGRNRISKTRRYRPG

***C. elegans* HCP-3/HCP-3L1 (CPAR-1) HFD alignment**

>C_elegans_HCP-3_HFD
QKALEEIRKYQKTEDLLIQKAPFARLVREIMQTSTPFGADCRIRSDAISALQEAAEAFLVE
MFEGSSLISTHAKRVTLMTTDIQLYRRLCLR
>C_elegans_HCP-3L1_CPAR-1_HFD
QKALEEIRKYQESEDLLIPKAPFARLVREIMQTSTPFSSDLRIRSDAINALQEASEALLVQM
FDGSSLISAHSKRATLTTTDVQLYRRLCLP

***C. panamensis* HCP-3/HCP-3L10 N-tail alignment**

>C_panamensis_HCP-3_N-tail
MLQMEDLDGPRIEELPASPEREPAALRDNNRNGNVQRALPAHFENELRRLMSDPNFSKD
AELMTDAIELMKRQVNKMEDDQDMYGYEGGMAEMIRNLRIRIVSFTKKRDDAIARFRE
EREASRQMERTRRVNNTDFDMTDAENRTRLHPSMSSQRSYSYDRRDPYSSDENDDTYQ
PARHNAQQRMSRSPSPLMHQSHHSSINQRRDDSRQHRSYNNTATSSRPTTSNRSRMRVG
KNCVTKTKNRKWKPG
>C_panamensis_HCP-3L10_N-tail
MLQMEDLDGPRNEEMPASPEREPAA----------
EAQFENELRRLMNDPNFHRKADLMGKAIELMKRQVNRLEDDQDVY----------
DD---------DASRLVERTRRVNNTDLDMTDAENRTRLRSSVSSQ------
RRDPYSSDENNDTDQPARHNAQQRMSRSPSSLMHQGHGSTINQRRDDNRRQRSYNNTA
TSSRPTTSNQSRMRIGKNRVTKTKNRKWKPG

***C. panamensis* HCP-3/HCP-3L10 HFD alignment**

>C_panamensis_HCP-3_HFD
EKAMKEIRRYQKSTDLLIQKAPFVRLVHEIMADVTPRSSEYRIRAEALGALQEAAEAFLV
EMFEGSVLIANHAKRVTLMPTDIQLYRRLCLR
>C_panamensis_HCP-3L7_HFD
KKAMNEIRRYQKSNDLLIQKAPFVRLVHEIMADVTPRSSEYRIRAEALGALQEAAEAFLV
EMFEGSMLIANHAKRVTLTPTDIQLYRRLCLR

***C. afra* HCP-3/HCP-3L8 N-tail alignment**

>C_afra_HCP-3_N-tail

MLHQHVGPVITEMEEPASRDSSILRDSGRRHNVARSEPTAIERQIQDIFNQPRCNERPEAM
AEAIRLMRKQIDQWEREQDLYGPTEERTKNIRIWKNNRRKFIAQLEVAKERQERARRER
DESSRVVETVRRRGDMTETNVTAIHNSTRLQSSQRSYDQRTHFDSDEEE--
NGYTSRAPRPLPRSPPRAHQSYHSNLSHQSRRLDASSQDNRSRNDYGSNNVTSVTSSSH
QGKPKPRLRAGKSRVTKNMFRPR
>C_afra_HCP-3L8_N-tail
MNNSRNTRLKSDIVPGRRIIPDVIYRDAGIEEEPSYLAERTMDESVVNSTRNTRWNSNIVP
GRRIIPDVIYRDSGIRGEPSYLAERTMDESVKSKRIGLGEREKRAADPTRSTMSSMIEDISS
PGVQFFENNREKMRPTVATPRRSNLGANRLSTASDKEKTIDMLSIGGEPTIEANGSSYVEP
VSVNGSRVSPTREDMLHRHVGPVITEVEESPSRDPSVLRDSGRRHNVARSKSTAIERQIKL
IFIEPRFKERPEAMAEVIRLMRKQIDQWERDQDSYGPTEERTKNIRAWKDNRRRFIEQLE
DAKERQERARRERDESSRVVETVRRRGDMTGMNVTAIHNS-------
RSYDQRTHFDSEEEEKENGYTSRAPRPLPRSPPRAHQSYHSSLSHQSRRLDASSRDNRSR
NDYGSNNV---TSSSHQGKPKPRLRAGKSRVTKSMFRPR

***C. afra* HCP-3/HCP-3L8 HFD alignment**
>C_afra_HCP-3_HFD
RDRALLEIRQYQKSTNLLIQKAPFCRLVQEILREVTSSSDYRSSDYRIRADALSALQEAAE
AFLVEMFEGSQLIATHARRVTLMHSDIQLYRRLCLR
>C_afra_HCP-3L8_HFD
RDRALLEIRQYQKSTDLLIQKAPFCRLVQEILREEATTS---
SSDYRIRADALTALQEAAEAFLVEMFEGSQLIATHARRVTLMHSDIQLYRRLCLR

***C. sp49* HCP-3/HCP-3L9 N-tail alignment**
>C_sp49_HCP-3_N-tail
MEMLLILILALLPPSSSTCLFSVLIISSSSSFPSNCTRLEGRFRVDHSSDLADSQLVELFANV
RELHGVVQIWNSRLTTANFLANIRRITGDLLDNSISIVNNTLLTSINLTSLEYSDGKVEIQN
NPLLDLKPNCPALHKSFFNRRSISSLECGCQVTGTFSYPKNINFPENCVVLYGNFIINDVAP
PFELLYRLLSVRKLYGFLEVRNTNLETLGFLQNLEEIESGPDDEITVSIGSEMTWLGRVDLI
KLNSIKSRNPRKSVISISAKCLDPALVQLVAHSDIQDIKPEMQRMDGPIIEEVVDHDARQE
KRNRRINRIKDLMARPETKESKELLKKMLDLLQEHLNDLEDEQLEEGANHRDAIATLRH
KIDIFTPLYNGAVADYMARKEESRREEARRAMSFGSSQNNITGRDNRSKLHQSSQRTYSS
EDEENDEEYAGRNRQDARRYHHQSPEPQASSSARRDNTRRVDASSNNPRMRAGKSRVT
KTNSRRWRPG
>C_sp49_HCP-3L9_N-tail

MARPETKESKELLKKMLDLLQEHLNDLEDEQLNEGANHRDAIATLRHKIDIFTPLYNAA
VADYMARKEESRREEARRAMSFGSSQNNITGRDNRSKLHQSSQRTYSSEDEENDEEYAG
RNRQDARRYHHQSPEPQASSSARRDHTRRVENSSNNPRMRAGKSRVTKTNSRRWRPG

***C. sp49* HCP-3/HCP-3L9 HFD alignment**
>C_sp49_HCP-3_HFD
QKALSEIRKYQKSTDMLIQKAPFHRVVQEILCETSGFTNAHRIRADAISALQEAAEAFLVE
MFEGAMLLSNHAKRVTLMASDIQLYRRLCLR

>C_sp49_HCP-L9_HFD
QKALSEIRKYQKSTDMLIQKAPFHRVVQEILCETSGFTNAHRIRADAISALQEAAEAFLVE
MFEGAMLLSNHAKRVTLMASDIQLYRRLCLR

Supplementary Data 3.S2: HCP-3 histone-fold domain codon based-alignments used for Figure 2

>C_tribulationis_hcp3_HFD
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CATGACTTTCGTATTCGTGCCGACGCTCTGATGGCTCTTCAAGAAGCATCTGAAGCGT
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CCTTATGCCGACTGATATTCAGTTGTACCGTCGTTTGTGTCTTCGA
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AAAGACTTTCGTATTCGTGCGGACGCTTTGATGGCTCTTCAGGAAGCCGCGGAAGCG
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Supplementary Data 3.S3: All HCP-3 sequences used in this study

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Supplementary Data 3.S4: HCP-3 protein motifs in C. elegans and C. sp54 found through manual alignments with sister species

***C. elegans* motif 1 in HCP-3/CPAR-1 aligned to *C. inopinata* motif 1**

>C_elegans_HCP-3_motif1_manuallyFound
-MADDTPIIEEIAEQNESVTR
>C_elegans_CPAR-1_motif1_manuallyFound
-MADDGPIIEEIAEKNGRVAR
>C_inopinata_HCP-3_motif1
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***C. sp54* motif 4 in HCP-3/HCP-3L3 aligned to *C. tropicalis* motif 4**

>C_sp54_HCP-3_motif4_manuallyFound
-----EELRDNLFEKMERAKADYYKRKEA
>C_sp54_HCP-3L3_motif4_manuallyFound
-----EETRDNYLEKMERAKAEYYKRKEA
>C_tropicalis_HCP-3_motif4
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Supplementary Data 3.S5: All HCP-4 sequences used in this study

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Supplementary Data 3.S6: All KNL-2 sequences used in this study

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Supplementary Data 3.S7: All KNL-1 sequences used in this study

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Supplementary Data 3.S8: All ZWL-1 sequences used in this study

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Supplementary Data 3.S9: All SPDL-1 sequences used in this study

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Supplementary Data 3.S10: All NDC-80 sequences used in this study

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Supplementary Data 3.S11: All HIM-10 sequences used in this study

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All supplementary data and supplementary tables are available in Caro L et al., 2022.

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Chapter 4. FUTURE DIRECTIONS

This chapter addresses lingering questions about the mechanism of *peel-1 zeel-1*. Section 4.1 focuses on the toxin mechanism while Section 4.2 focuses on the antidote mechanism. Both sections highlight multiple unanswered questions and experiments probing these questions. The data shown here are preliminary, and therefore, results should be cautiously interpreted. Section 4.3 explores evolutionary questions regarding *peel-1 zeel-1*, including its possible origins.

My model of how the *peel-1 zeel-1* element works is the following. PEEL-1 is loaded into sperm and does not cause toxicity in these cells because they are missing PMPL-1. After fertilization, PEEL-1 protein is delivered to the embryo. PEEL-1 on its own forms an oligomer, where amphipathic helices construct the lining of the channel. However, the PEEL-1 channel is in a closed conformation when PMPL-1 is not present. The PEEL-1 channel remains in a closed and inactive conformation during early embryonic development because of missing (or low levels) of PMPL-1. Soon after the maternal-to-zygotic transition, *zeel-1(+)* embryos express the antidote, resulting in interactions between the antidote (ZEEL-1), PEEL-1, and E3 ubiquitin ligase machinery. As a result of these interactions, PEEL-1 is ubiquitylated and degraded. On the other hand, *zeel-1(-)* embryos do not degrade PEEL-1 and the toxin is maintained in the muscle and epidermal tissues. In the two-fold stage of embryonic development, PMPL-1 levels are high enough in the muscle and epidermis to cause toxicity in these tissues. PMPL-1 interacts with the PEEL-1 cation channel, gating the channel open, causing influx of sodium, osmotic stress, cell swelling, cell death, and finally, death of the embryo.

4.1 MECHANISM OF PEEL-1 TOXICITY

In my model, I hypothesize that oligomers of PEEL-1 amphipathic helices are the primary structural component of the toxic cation channel. I hypothesize that PMPL-1 interacts with a PEEL-1 oligomer, causing a conformational change in the channel to gate it open, allowing passage of monovalent cations through the membrane.

4.1.1 Do PEEL-1 and PMPL-1 interact?

To test the hypothesis that PEEL-1 toxicity involves a direct protein-protein interaction between PEEL-1 and PMPL-1, I have carried out several investigations, but results have varied. In some cases, I have been unable to detect an interaction between PEEL-1 and PMPL-1. These negative results may be because of technical challenges, namely, PEEL-1 and PMPL-1 co-expression causes cell death and the assays I have used are prone to false-positive results. Meanwhile, in other cases, I have detected an interaction between PEEL-1 and PMPL-1, but the results are difficult to interpret without stringent negative controls. Here I present data from assays probing PEEL-1 and PMPL-1 interactions in yeast, mammalian cells, worms, *E. coli*, and with purified proteins *in vitro*.

Mating-based split ubiquitin system (mbSUS) is an assay in yeast used to test for protein-protein interactions *(Horaruang & Zhang, 2017)*. Ubiquitin is split into two fragments (named Nub and Cub) and fused to proteins of interest. The Cub fragment is also fused to a transcription factor, LexA. Haploid strains of either Nub or Cub are generated, mated together, and assayed for growth on -his medium. This assay works because an interaction between the fused proteins results in reconstitution of the ubiquitin moiety, release of the LexA transcription factor, and expression of the gene HIS3, required for growth in -his medium. Therefore, growth on -his is indicative of a protein-protein interaction.

mbSUS strains expressing Cub-tagged PEEL-1 and Nub-tagged PMPL-1 did not grow on -his media, indicating these proteins did not interact (Fig. 4.1A and 4.1C). However, the fact that I could easily generate this strain and that it grows normally suggests that PEEL-1 and PMPL-1 do not kill yeast. This lack of killing activity raises the question of whether the reason these proteins do not kill yeast is the same reason why they do not interact in mbSUS, possibly due to yeast missing a co-factor for interaction and toxicity (e.g., a specific lipid). Alternatively, the yeast cell wall may suppress the cell swelling induced by toxicity or the tags may disrupt the functions of PEEL-1 and PMPL-1. Major results and interpretations are outlined below (Table 4.1).

Table 4.1. Results and interpretations from mbSUS for PEEL-1 and PMPL-1 interaction

Strain #	Cub construct	Nub construct	Growth on -his	Interpretation
1810	PEEL-1::Cub	NubG::PMPL-1	No	No interaction
1814		PMPL-1::NubG	No	No interaction
1851	PEEL-1(-94aa)::Cub	NubG	No	Not self-activating
1920		NubG::PMPL-1	Yes	True interaction?*
1846		NubG::PMPL-2	Yes	True interactor? Not a good negative control.
1728	PMPL-1::Cub	NubG	Yes	Self-activating Cub
1726		NubG::PEEL-1	Yes, but less than NubG	Ambiguous. Better negative control necessary
1731		PEEL-1::NubG	Yes, but less than NubG	Ambiguous. Better negative control necessary

*PEEL-1(-94aa)::Cub interacts with other constructs that wild-type PEEL-1::Cub does not interact with, including PMPL-1, PMPL-2, GADR-5(TM), and ZEEL-1(4TM) (see section on ZEEL-1 PEEL-1 mbSUS). This interaction suggests that removing over half of the PEEL-1 protein allows it to interact with more proteins than the wild-type PEEL-1. While this counterintuitive result be biologically relevant, it introduces some caution to interpreting data from this construct.

Bimolecular fluorescence complementation (BiFC) experiments can also test for interactions between proteins. The Venus fluorophore is split in two pieces (Venus-N and Venus-C) and fused to proteins of interest. The split-Venus fragments are not fluorescent on their own, but when in close proximity, a fluorescent, bimolecular complex is formed, with fluorescence read-out of protein-protein interaction. BiFC experiments have been performed mostly in HEK293T cells and complicates results when working with full-length proteins, since PMPL-1 and PEEL-1 kill these cells. To test for interaction between these proteins in cells that do not die, I have used non-toxic mutants of PEEL-1 and PMPL-1.

Since BiFC is known to have false-positive interactions, I attempted to control interactions between PEEL-1 and PMPL-1 by testing PEEL-1 with PMPL-1 paralogs (PMPL-2 and PMPL-7). A PEEL-1 loss-of-function mutant (D109A) interacts more with PMPL-1 than PMPL-2 (Fig. 4.2A). However, the difference in fluorescence is marginal, making interpretation of this result difficult. I also found that wild-type PEEL-1 interacts more with a truncated PMPL-1 (containing only C-terminal residues 28-59) than corresponding PMPL-2 and PMPL-7 truncations (based on alignments in Chapter 2) (Fig. 4.2B). This C-terminal PMPL-1 interaction with PEEL-1 is notable, since LDH assays using PMP3-like chimeras suggest that this same C-terminal region of PMPL-1 is sufficient for toxicity, as long as it is fused to any PMP3-like N-terminal half (discussed later) (Fig. 4.6B-C). A major caveat is that I have not tested whether PMP3-like::Venus-C constructs express at similar levels. Varied expression levels of PMP3-like constructs could explain these data.

I attempted co-immunoprecipitation experiments in HEK293T cells to assay PEEL-1 and PMPL-1 interaction but did not get a positive result. Here, an antibody targeting one protein allows isolation, i.e., immunoprecipitation (IP), of the target protein and co-immunoprecipitation

(co-IP) of the proteins it interacts with. Using an anti-GFP nanobody, I could successfully IP PEEL-1::eGFP. However, I was unable to detect co-IP of PMPL-1::mCherry (data not shown). This result may suggest PEEL-1 and PMPL-1 do not interact. However, co-IP experiments often must be optimized for membrane proteins. I used Nonidet P40 detergent to lyse cells, but this detergent may disrupt the very interactions I was hoping to detect. Alternatively, the interaction between PEEL-1 and PMPL-1 may be short-lived, so the use of crosslinkers prior to co-IP may be necessary. The cell death caused by PEEL-1 and PMPL-1 could also make it difficult to co-IP these proteins from HEK293T cells, so using loss-of-function mutants for co-IP experiments should be considered. The mutants used must be carefully chosen to ensure that the hypothesized protein-protein interactions are preserved.

4.1.2 Does PEEL-1 oligomerize?

My model of toxicity involves homotypic interactions among PEEL-1 proteins. mbSUS experiments did not support a homotypic PEEL-1 interaction (Fig. 4.3A). However, western blots of whole-cell lysate from bacteria expressing PEEL-1 with a maltose-binding protein tag (MBP) showed a high molecular weight band at about the expected size for an MBP::PEEL-1 dimer (Fig. 4.4A). Since whole-cell lysates have all the endogenous bacterial proteins, it seems unlikely the dimer band is due to PEEL-1 nonspecifically aggregating with proteins. The defined dimer band in whole cell lysate samples suggests a specific homotypic PEEL-1 interaction. After purifying MBP::PEEL-1, these dimer bands can still be seen (Fig. 4.4A-B). Interestingly, mass photometry of PEEL-1 in beta-octylglucoside detergent (β-OG, used for purification), does not show a dimer (Fig. 4.4C). If I instead dilute this same PEEL-1 in SDS, mass photometry shows that PEEL-1 oligomerizes (Fig. 4.4D). Therefore, SDS may induce the oligomerization of PEEL-

1. Since SDS is negatively charged, this SDS-induced oligomerization may suggest that negatively charged lipids permit or induce PEEL-1 oligomerization in cell membrane. However, whether these oligomers exist *in vivo* is still unknown.

One possible approach to testing oligomerization in HEK293T cells is through total internal reflection fluorescence (TIRF) microscopy, combined with single molecule photobleaching. By counting the number of photobleaching steps of a PEEL-1 complex at the plasma membrane, the oligomeric state of this protein in cells could be determined *(Senning and Gordon, 2015)*. However, this experiment may be difficult to interpret if PEEL-1 does not have a defined oligomeric state.

4.1.3 Does PMPL-1 oligomerize?

The molecular functions of PMPL-1 remain unknown, but its roles may involve homotypic interactions. I found that western blots of bacteria expressing MBP::PMPL-1 result in a band at the expected size of a homotypic dimer in both bacterial whole-cell lysate (Fig. 4.5A) and purified protein (Fig 4.5B). Mass photometry of purified MBP::PMPL-1 in beta-octylglucoside detergent shows multiple higher-order oligomers (Fig. 4.5C). Similar oligomers are seen with other detergents (lauryl maltose neopentyl glycol, n-decyl-beta-maltoside, and n-dodecyl-beta-maltoside), although to varying degrees (Fig. 4.5D). This detergent-dependent oligomeric state could suggest that PMPL-1 oligomerization is dependent on its nearby lipid and protein environment. Alternatively, this could be nonspecific aggregation *in vitro*, especially since mass photometry requires diluted samples which also dilute the detergent. It is still unclear whether these oligomers exist *in vivo* or whether it is important for PMPL-1's role in PEEL-1 toxicity.

mbSUS experiments for PMPL-1 homotypic interactions suggest that PMPL-1 interacts with itself, as well as PMPL-2 (Fig. 4.3B). I am cautious in interpreting this result as PMPL-1 interacting with itself and with PMP3 proteins broadly, since I have seen PMPL-1::Cub interacts with many membrane proteins, shown in Fig. 4.8C.

4.1.4 Which residues are critical for PMPL-1's role in toxicity?

Although PMPL-2 is 75% similar to PMPL-1 (Fig. 4.6A), it does not kill HEK293T cells when co-expressed with PEEL-1. Chimeras between these closely related PMP3-like proteins can therefore be used to determine which residues are important for toxicity. I found that the C-terminal half of PMPL-1 (PMPL-1(aa27-59)) is most important for toxicity (Fig. 4.6B). Expression of the C-terminal region alone is not sufficient for toxicity with PEEL-1. However, fusing this PMPL-1(aa27-59) to the N-terminal halves of either PMPL-2, PMPL-7, or yeast PMP3 allows for toxicity with PEEL-1 (Fig. 4.6C). These data suggest a model where the C-terminal end of PMPL-1 allows for specific interaction with PEEL-1, and the N-terminal end provides some important structural feature to the protein. This model is corroborated by preliminary BiFC data presented earlier (Fig. 4.2B), where PEEL-1 interacts more with PMPL-1(aa29-59) than with C-terminal truncations of other PMP3-like proteins.

Future directions

I have already outlined some future directions which test the main mechanistic hypotheses (see above). Below I discuss possible directions to further probe ion channel activity, protein interactions, and pore-lining residues.

4.1.5 An alternative approach for assaying the PEEL-1/PMPL-1 ion channel

My previous experiments used planar lipid bilayers to test for PEEL-1 and PMPL-1 ion channel activity (see Chapter 2). Planar lipid bilayers are a powerful tool to study ion channels since single channel activity can be measured. However, planar lipid bilayer experiments are time consuming, are variable day-to-day, and require extensive technical expertise. Finding an alternative assay that is higher throughput can help validate previous experiments and address new biochemical questions like: What is the ion selectivity series of the channel? Does mutating the putative selectivity filter (D109) change ion selectivity? Are there specific lipids that promote ion channel activity?

A liposome flux assay is a possible alternative approach to *in vitro* PEEL-1 and PMPL-1 experiments *(Su et al., 2016)*. This approach sacrifices the single channel resolution provided by planar lipid bilayer. Therefore, the liposome flux assay can average out variability, between single proteins or channels, making results easier to interpret. Liposome flux assays have been used to test for activity of channels conducting potassium, chloride, and protons *(Su et al., 2016, Cabanos et al., 2017, Dickson et al., 2014, Liu et al., 2023)*. Liposome flux assays rely on an intraliposomal, pH sensitive fluorophore impermeable to lipid bilayers, AMCA. The experiment works in four steps: (i) equilibration of ion channel-containing liposome in a buffer with a non-permeable cation, (ii) addition of a proton ionophore (CCCP) to allow for the passage of protons into the liposome, (iii) movement of ions from the lumen of the liposome into the extra-liposomal buffer, and (iv) countermovement of protons into the liposome, thus acidifying the intraliposomal solution and quenching of the fluorophore. Therefore, measuring fluorescence over time provides a proxy for ion movement. Addition of monensin at the end of the assay

allows a path for sodium to travel through the liposome, thus providing a maximum fluorescence quenching for normalization of fluorescence data.

I attempted to use the liposome flux assay to test for ion channel activity of PEEL-1 and PMPL-1, but I faced many technical challenges, possibly due to the many moving pieces in this assay. I created liposomes containing PEEL-1 alone, PMPL-1 alone, both proteins together, and no proteins. I used Na^+ as the permeable cation inside the liposomes and diluted liposomes in buffer containing $NMDG^+$, the impermeable cation. All protein-containing liposomes caused significant fluorescence quenching, suggesting that both PEEL-1 alone and PMPL-1 alone allow for Na^+ to leave the liposomes (Fig. 4.7A-B). Furthermore, even before the addition of CCCP, some experiments showed significant quenching (Fig. 4.7B), suggesting that these proteins allow H^+ conduction through the liposomes. Liposomes containing both proteins did often quench more than each protein alone, but this result is difficult to interpret since the intended negative controls do not behave as expected (Fig. 4.7A-B). The amount of quenching from aliquots of the same liposome prep was variable depending on the day and the material of the 96-well plate used (for example, polystyrene or polypropylene). Some possible interpretations of the data from these experiments are described in Table 4.2.

Table 4.2. Interpretations and counter arguments explaining results from liposome flux assays.

Possible interpretation	Counterargument
PMPL-1 weakens the liposome, allowing ions through	Acidification of a compartment is required for fluorescence quenching. Since we see quenching, it suggests there is an isolated compartment in this assay.
PEEL-1 creates a channel on its own	None. But means that additional controls may be needed, such as PEEL-1(-65aa).
PMPL-1 creates a channel on its own	None. But means that additional controls may be needed, such as PMPL-2.

The lipid composition of the liposome is sub-optimal (50% DOPC/POPS)	I repeated the experiment once, using soybean lipid extract with cholesterol, and saw similar results in the liposome assay.
Technical issue. I have seen variability depending on the material of the plate	None. Need a good positive and negative control for this experiment, with proteins known to/not conduct ions through a liposome.
The experiment is measuring something other than ion movement through the liposome.	Diluting liposomes in Na^+ or K^+ instead of the impermeable cation $NMDG^+$ result in minimal quenching. Therefore, quenching only occurs when the extraliposomal buffer contains an impermeable cation.

Other approaches are possible to test for ion flux through liposomes *in vitro*. One approach is incorporating membrane-impermeable, fluorescent ion indicators into liposomes. This fluorescence read-out provides a more direct measure of ion flux through liposomes compared to my current flux assay. Similar approaches have been used for Zn^{2+} channels *(Gati et al., 2017)*. A different approach uses radioactivity to measure ion movement into liposomes, using either $^{22}Na^+$ for sodium channels (Villegas et al., 1980) or $^{86}Rb^+$ for potassium channels *(Nimigean 2006)*. Radioactivity may be the most direct approach for measuring ion movement into liposomes. However, a disadvantage to this approach is that it requires running samples through a cation exchange column in order to specifically measure intraliposomal radioactivity, making this an endpoint rather than a kinetic assay. In personal communications with Jakub Sliwinski (TraceLab, UW Oceanography), one possible approach is by using non-radioactive Rb and inductively coupled mass spectrometry (ICP-MS) as a readout for Rb concentration in liposomes.

4.1.6 Determine important residues in PEEL-1 and PMPL-1

Deep-mutational scans (DMS) can be used to identify important residues in a protein. This approach is versatile since selection on the DMS library can be modified to test different aspects of a model. For example, to determine which PEEL-1 residues are important for toxicity, HEK293T cells expressing PEEL-1 DMS variants can be grown in bulk. By inducing PMPL-1 expression, functional variants cause cell death and are lost from the population over time. By sequencing pools of cells after several generations, you would expect an enrichment of PEEL-1 mutants which have lost their toxic activity. This would be a great system to thoroughly test whether the amphipathic property of the PEEL-1 AH correlates with toxicity. DMS can also be used to test for interactions between PEEL-1 and PMPL-1. Here, DMS can be coupled with BiFC, and fluorescence can be used as a selective force by fluorescence-activated cell sorting. This would allow us to find mutants which increase or decrease protein-protein interactions. Similar approaches can be used to test for homotypic protein interactions.

4.1.7 Determine the pore-lining residues of PEEL-1

I have data consistent with the PEEL-1 amphipathic helix constructing the lining of the cation channel, but this hypothesis can be tested more rigorously. The substituted cysteine accessibility method (SCAM) can be used to determine the pore-lining residues of an ion channel *(Liapakis et al., 2001)*. It relies on the principle that adding a bulky residue to the lining of the channel will change a specific channel property. Importantly, the bulky residue cannot abolish channel activity entirely, nor just symmetrically reduce its current. Either the channel rectification or ion selectivity must be altered to support the model. Bulky residues can be reversibly introduced by adding a thiol reagent such as 2-[(methylsulfonyl)thio]-ethanesulfonic acid (MTSES) which will covalently bind to cystines that are accessible from the extracellular

solution. A down-side to these experiments is that they would likely need to be performed using electrophysiology, which can be technically challenging because of the cell swelling and death caused by PEEL-1 and PMPL-1.

4.2 MECHANISM OF ZEEL-1 ANTIDOTE ACTIVITY

My current model of antidote activity is that ZEEL-1 and PEEL-1 interact through their transmembrane domains, resulting in ubiquitylation and degradation of PEEL-1. Although I have reconstituted antidote activity in HEK293T cells (see Chapter 2), the precise mechanism of antidote activity remains unknown.

4.2.1 Do PEEL-1 and ZEEL-1 interact?

My model of ZEEL-1 antidote activity involves a direct protein-protein interaction between PEEL-1 and ZEEL-1. mbSUS experiments support this model, showing that PEEL-1 interacts with both full-length ZEEL-1 and a truncated ZEEL-1 with only contains the transmembrane domains (ZEEL-1(TM)) (Fig. 4.8A). PEEL-1 interaction with ZEEL-1 is specific, since PEEL-1 does not interact with GADR-5(TM) in this assay (the most similar ZEEL-1 paralog) (*Seidel et al., 2011*) (Fig. 4.8A). Removing the last two ZEEL-1 TM domains eliminates its interaction with PEEL-1 (Fig. 4.8B), illustrating their requirement for this interaction. I hypothesize that the interaction between PEEL-1 and ZEEL-1 is required for antidote activity, but I have not tested this.

In addition to ZEEL-1 interaction with PEEL-1, it is possible that it also interacts with PMPL-1 as part of its antidote mechanism. However, mbSUS experiments that test ZEEL-1 interactions with PMPL-1 are difficult to interpret. PMPL-1::Cub interacts with both ZEEL-

1(TM) and GADR-5(TM) (Fig. 4.8C), as well as most other membrane proteins I have tested. This broad interaction might suggest that PMPL-1::Cub is prone to false-positive interactions. Alternatively, if these results are biologically meaningful, PMPL-1 may indeed interact broadly with membrane proteins. Differentiating between these two possibilities requires more experiments and possibly different assays. A summary of results is shown in Table 4.3.

Table 4.3. Results and interpretations from mbSUS for ZEEL-1 interactions.

Strain #	Cub construct	Nub construct	Growth on -his	Interpretation
1785	PEEL-1::Cub	NubG	No	No self-activation of Cub
1806		NubG::ZEEL-1	Yes	Interaction
1827		NubG::ZEEL-1(TM)	Yes	Interaction in TM domains
1887		NubG::GADR-5(TM)	No	No interaction. PEEL-1 interaction is specific to ZEEL-1
1914		NubG::ZEEL-1(4TM)	No	Last two TM domains of ZEEL-1 are required for interaction
1728	PMPL-1::Cub	NubG	Yes	Self-activating Cub
1828		NubG::ZEEL-1(TM)	Yes (more than NubG)	Interaction
1888		NubG::GADR-5(TM)	Yes (more than NubG)	Interaction. Not a good negative control?

4.2.2 Does ZEEL-1 ubiquitylate PEEL-1 for degradation?

My current model of antidote activity involves ZEEL-1-mediated ubiquitylation and degradation of PEEL-1. I have not tested this hypothesis, but there are several avenues to do so. I have already shown that mCherry::ZEEL-1 suppresses toxicity from PEEL-1::eGFP in HEK293T cells. Flow cytometry or western blots can be used to measure levels of PEEL-1::eGFP when transfected alone or in combination with mCherry::ZEEL-1. Any effect from co-

transfection can be controlled for by using mCherry::GADR-5, which does not suppress toxicity. The addition of a proteasome inhibitor like MG132 should prevent any proteasome-mediated degradation. Furthermore, finding that E3 ubiquitin ligase machinery can co-IP with ZEEL-1 would suggest that ubiquitylation is involved in the antidote mechanism. Similar co-IP experiments have been successful with GADR-5 in HEK293T cells *(Sawyer et al., 2011)*. An alternative hypothesis of the antidote mechanism may be that ZEEL-1 acts by disrupting homotypic PEEL-1 interactions or interactions between PEEL-1 and PMPL-1.

4.2.3 Deep mutational scan to determine interacting residues between PEEL-1 and ZEEL-1

mbSUS can be a platform for further probing the interaction between PEEL-1 and ZEEL-1. Mutants in either protein which reduce their interaction should result in lack of growth on assay plates (-his). In theory, these mbSUS experiments can be scaled-up and mutants can be assayed in bulk, using sequencing to assay for relative growth of mutants. This approach may find missense mutations that eliminate the interaction between ZEEL-1 and PEEL-1. Determining whether these same mutants result in loss of antidote activity in HEK293T cells or in *C. elegans* would allow us to more rigorously test whether interactions between PEEL-1 and ZEEL-1 are required for antidote activity.

4.3 EVOLUTION OF *PEEL-1 ZEEL-1*

Although we understand how many selfish genetic elements spread in populations, we often do not know how they first evolved. I hypothesize that animal TA systems face many hurdles during their evolution, requiring more complexity than fungal or prokaryotic TA systems.

These hurdles include evolving germline-specific expression of the toxin, as well as preventing toxicity in off-target cells.

4.3.1 Where did *peel-1 zeel-1* come from?

The evolutionary origin of *peel-1 zeel-1* remains a mystery. The *peel-1 zeel-1* TA has only been found in *C. elegans* and is absent in many strains within this species *(Seidel et al., 2008, Seidel et al., 2011)*. Both PEEL-1 and ZEEL-1(TM) are evolutionarily novel, and their evolution within *C. elegans* cannot be obviously traced. How did these genes evolve? An intriguing idea is that they were horizontally transferred from another organism. Horizontal gene transfer events are rare and often likely lost by genetic drift, but selfish activity can overcome this evolutionary hurdle. Recent work also provides a precedent for horizontal gene transfer of a toxin-antidote system in animals *(Widen et al., 2023)*. Finding transposable elements which flank the *peel-1 zeel-1* genomic locus may lend support to this hypothesis, but this has not been found. Without a DNA sample from another organism which shows homology to *peel-1 zeel-1*, it would be difficult to confidently determine whether these genes evolved *de novo* in *C. elegans* or whether they were horizontally transferred from another organism.

4.3.2 Why does PEEL-1 rely on another protein for toxicity?

One of the simplest mechanisms for a population to gain resistance to the *peel-1 zeel-1* selfish element might be by mutating *pmpl-1*. *pmpl-1* mutant worms are perfectly viable in laboratory conditions, so this may be a feasible evolutionary route to suppressing the TA (although the high conservation of *pmpl-1* suggests it is important in nature). But might there be some benefit for *peel-1 zeel-1* to require a second protein for its selfish activity? Although PEEL-

1 toxicity is critical for the success of the selfish element, the precise timing of toxicity is important. I hypothesize that relying on PMPL-1 allows for precise developmental timing of PEEL-1 toxicity.

There are two critical times where PEEL-1 protein should not kill: the sperm and the early embryo. Sperm carry the toxin to the progeny, so sperm viability is important to the success of the selfish element. Early embryonic development is mostly guided by maternally deposited transcripts. PEEL-1 toxicity must occur after the maternal-to-zygotic transition to allow time for zygotic expression of ZEEL-1. A route to prevent off-target killing in both cases is possible by requiring a second component that is only present in the target developmental stage or tissue. All of this means that PMPL-1 may not have been co-opted by PEEL-1 because of its endogenous functions, but instead, because of the developmental timing and tissues in which it is (and is not) expressed. Just PEEL-1's ability to detect the presence of PMPL-1 can serve as a trigger for PEEL-1 to turn from dormant to toxic. The *sup-35 pha-1* toxin-antidote system in *C. elegans* also requires genes that are expressed in specific tissues *(Ben-David et al., 2017)*. Tight control of the timing of toxicity may be a hurdle that TA systems must evolve in order to be successful genetic parasites. Co-option of endogenous proteins may be a common and evolutionarily accessible path for preventing off-target toxicity in animal toxin-antidote systems. Therefore, studying the mechanisms of toxin-antidote systems can provide insights into the origins and evolution of toxin-antidote systems.

4.4 METHODS

4.4.1 Mating-based split ubiquitin system (mbSUS)

mbSUS strains (gift from Dana Miller) were generated in haploid yeast, strain THY.AP4 (for Cub constructs) or strain THY.AP5 (for Nub constructs). Strains were streaked onto YPD plates, grown at 30°C for two days, and scraped off plates for transformation in PLATE solution (40% PEG, 100 mM LiOAc, 10mM Tris-HCl 7.5, 1mM EDTA). 10µL of 10µg/mL boiled salmon sperm DNA was added to cells, along with ~600ng of the target construct, and cells were vortexed. After 1-6 hours at room temperature, cells were heat shocked in a 42°C water bath for 15 minutes. Cells were pelleted (1,000 x *g*, 1 min), washed with sterile water, and plated on selective agar media, either -leucine (-L) for Cub constructs or -tryptophan (-W) for Nub constructs. Plates were placed at 30°C for 2-3 days. Multiple colonies were pooled together, grown in appropriate selective liquid medium (overnight, 30°C), and frozen at -80°C for later use.

To generate the desired diploid strain to test for interactions between Nub-tagged and Cub-tagged proteins, haploid Nub strains were mated with haploid Cub strains. 1mL of overnight Nub or Cub cultures (30°C) were pelleted (1,000 x *g*, 5 min) and resuspended in 100µL YPD. 10µL of Cub culture was mixed with 10µL of Nub culture, spotted onto YPD agar plates, and incubated at 30°C for 6-8 hours. Cells were then scraped off the plate and used to inoculate 4-5mL of selective liquid media (-WL). Cultures were place in a 30°C shaker overnight prior to the spot assay.

To assay diploid strains for growth on different media, a spot assay was used. The density of overnight liquid cultures (30°C, shaking) was determined by measuring the OD_{600} of a 1:10 dilution of the culture in water. In parallel, cells were pelleted (2,000 x *g*, 2 min) and resuspended in water to a final $OD_{600} = 1.0$. Serial dilutions (10-fold) were made and spotted (5µL) onto multiple plates. Plates lacking tryptophan and leucine (-WL) were control plates and

plates also lacking histidine (-WLH) were assay plates. Different methionine concentrations were also tested since increasing methionine concentrations reduces expression of the Cub construct. Plates were incubated at 30°C for 2-3 days before growth was recorded and imaged.

4.4.2 Bimolecular fluorescence complementation (BiFC)

BiFC experiments were performed using transient transfection in HEK293T cells, followed by flow cytometry. Approximately $7x10^4$ cells were plated in each well of a 12-well plate ~24 hours prior to transfection. Transfections were performed using combinations of two BiFC constructs (one Venus-N tagged, one Venus-C tagged, 750ng each, total 1.5µg DNA with 3µg PEI). 40-48 hours after transfection, culture supernatant was removed, and adhered cells were collected for flow cytometry. Cells were resuspended by 5mM EDTA in 1X PBS (rocked for 10 min), pelleted in microcentrifuge tubes (500g for 5 min), and washed with 1X PBS. After resuspension in 1X PBS, cells were subjected to flow cytometry (Symphony S6). Forward scatter, side scatter, and YFP fluorescence was measured for 10,000 events. Gating was performed based on forward scatter and side scatter, although raw, ungated datasets showed similar results to gated datasets. Histograms were generated at floreada.io.

All constructs were made in an mCherry_N1 backbone with stop codons after the BiFC construct, followed by mCherry. This downstream mCherry is expressed at low levels. This was not intentional. mCherry_N1 alone was tested for fluorescence in this experiment, and I confirmed it did not emit in the YFP channel in flow cytometry.

4.4.3 Mass photometry

Mass photometry experiments were performed on a Refyn Two MP. MBP::PEEL-1::His_8 and MBP::PMPL-1 were purified as described in Chapter 2, resulting in purified protein in Buffer A (HEPES pH 7.4, 150mM NaCl, 5mM 2-mercaptoethanol, 10% glycerol) with beta-octylglucoside detergent (1%) and maltose (10mM). Prior to each experiment, glass slides were washed with water and isopropanol, and dried under a nitrogen stream. Oil (Immersol 518F) was spotted onto the lens and the glass slide was added on the oiled lens. 18µL of 0.2-µm filtered Buffer A (without detergent) was spotted onto the glass slide and put into focus using the "drop dilution" mode on the Acquire MP software. A 1-minute movie was recorded to confirm low background signal. ~2µL of protein was added directly to the buffer on the slide, mixed by pipetting, and the movie was immediately recorded. A target final protein concentration of approximately 50nM was used. Serial dilutions were performed in Buffer A when necessary. Discover MP software was used to generate histograms and Gaussian fits to the data. Molecular weights were determined based on a standard curve generated by beta-amylase (BAM) protein.

4.4.4 Liposome flux assay

Proteo-liposomes were made with MBP::PEEL-1::His_8, MBP::PMPL-1, or both. Proteo-liposomes were made as described in Chapter 2, but an increased salt concentration was used during purification and liposome formation (400mM NaCl). Proteo-liposome lipid compositions were either 50% DOPC:POPS (1,2-dioleoyl-sn-slycero-3-phosphocholine: 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine) or soybean lipids (Avanti Polar Lipids) with cholesterol. After liposome formation, flotation, and collection (see Chapter 2), liposomes were snap frozen in liquid nitrogen and kept a -80°C. Liposomes were thawed fresh before each experiment. Outside flux buffer was made by mixing Buffer B (20mM HEPES pH 7.4, 400mM N-methyl-D-

glucamine, 1mM EDTA) mixed with ACMA (in DMSO) (final concentration 2μM ACMA) before each experiment. 190μL of Outside flux buffer was added to a well of a black 96-well plate (black, 96-well, conical bottom, Nunc catalog #249945). Fluorescence was measured (excitation 419nm, emission 490nm, on a Spectra Max Gemini XPS) for 1 minute in 20-second (or 30-second) intervals, and 10uL of proteo-liposomes were added and mixed by pipetting. Fluorescence was measured in 20-second intervals until fluorescence levels stabilized. 1μL of 0.4mM CCCP (in DMSO) was added and mixed by pipetting. Fluorescence was again measured in 20-second intervals until fluorescence levels stabilized. 1μL of 5mM monensin (in DMSO) was added and mixed. Fluorescence was measured for 1 minute in 20-second intervals. Each fluorescence data point was normalized using the following calculation: normalized fluorescence = (fluorescence at data point – final fluorescence) / (initial fluorescence – final fluorescence). Two time points could be used for initial fluorescence, either fluorescence prior to liposome addition or fluorescence prior to CCCP addition. Data points after monensin addition were averaged to get a value for final fluorescence.

4.5 FIGURES

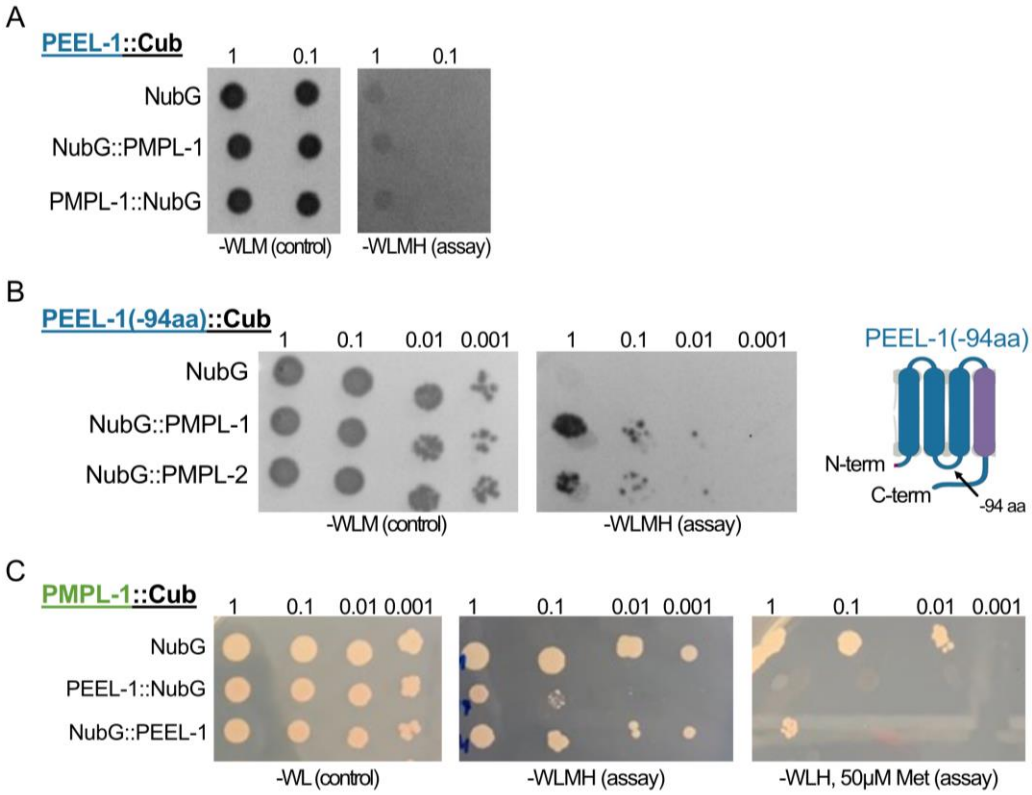

Figure 4.1. mbSUS assaying PEEL-1 and PMPL-1 interactions.

(A-C) Three experiments assaying PEEL-1 and PMPL-1 interaction using mbSUS in yeast. Yeast with indicated Cub construct were mated to yeast with indicated Nub construct (NubG is an untagged control). Resulting diploids were spotted on agar media with the indicated amino acids absent from the media (bottom; "-", W = tryptophan, L = leucine, M = methionine, H = histidine). 10-fold serial dilutions (ex. 0.1 indicates a 1 in 10 dilution) were spotted and an image of their growth is shown after 2-3 days at 30C. Left panels: -WL or -WLM = control (selects for Cub and Nub plasmids). Right panels: -WLMH = assay plates. All experiments were repeated at least three times and had similar results. **(B)** A truncated, PEEL-1(-94aa) Cub construct is used. Diagram on right shows the predicted structure of PEEL-1. indicated the -94aa. The -94aa truncation removed the PEEL-1 amphipathic helix (purple) and the third transmembrane domain.

(C) PMPL-1::Cub assayed with PEEL-1, showing growth in negative control (NubG). Increased methionine (M) concentrations reduce expression levels of Cub constructs. Two assay plates are shown, -WLMH and -WLH(50μM Met).

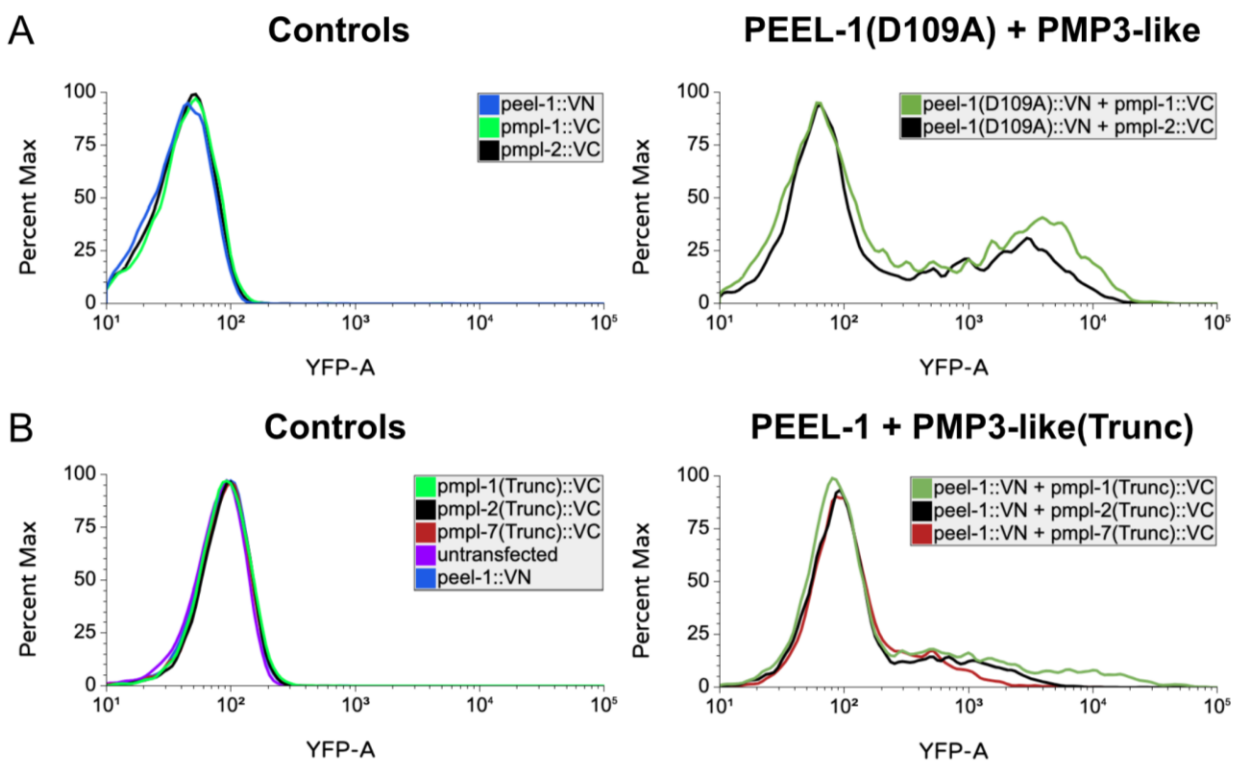

Figure 4.2. BiFC assaying PEEL-1 and PMPL-1 interactions.

Two examples of BiFC experiments performed in HEK293T cells to test for PEEL-1 and PMPL-1 interaction. Bimolecular fluorescence of Venus was assayed using a flow cytometry about 41 hours after transfection. Histograms of yellow fluorescence (YFP-A, x-axis) are shown as a percent max of the largest peak within each dataset (Percent Max, y-axis). Each construct is shown transfected alone (left, control) or together (right). **(A)** PEEL-1(D109A)::Venus-N tested for interaction with indicated PMP3-like::Venus-C. **(B)** PEEL-1::Venus-N interaction tested with indicated PMP3-like(Trunc)::Venus-C. Truncated, C-terminal-only of PMP3-like protein corresponds to PMPL-1(aa27-59) in alignments.

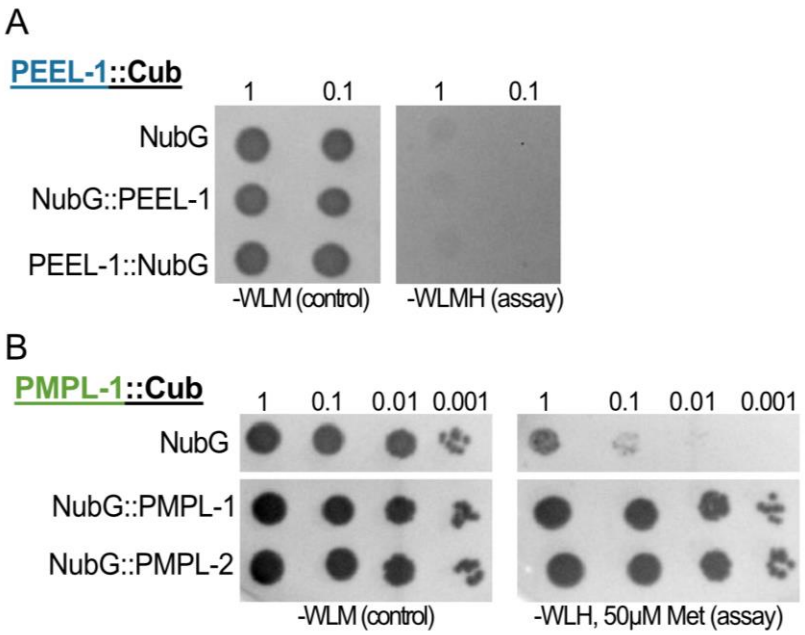

Figure 4.3. mbSUS for homotypic PEEL-1 and PMPL-1 interactions.

mbSUS experiments assaying homotypic interactions in **(A)** PEEL-1 and **(B)** PMPL-1. Yeast with indicated Cub construct were mated to yeast with indicated Nub construct and serial dilutions were plated. Control plates (-WLM) and assay plate (-WLH, with either -M or 50µM Met) are shown. **(A)** No growth is seen in assay plates of these diploids. **(B)** PMPL-1::Cub interacts with NubG::PMPL-1 and NubG::PMPL-2. All experiments were repeated at least three times.

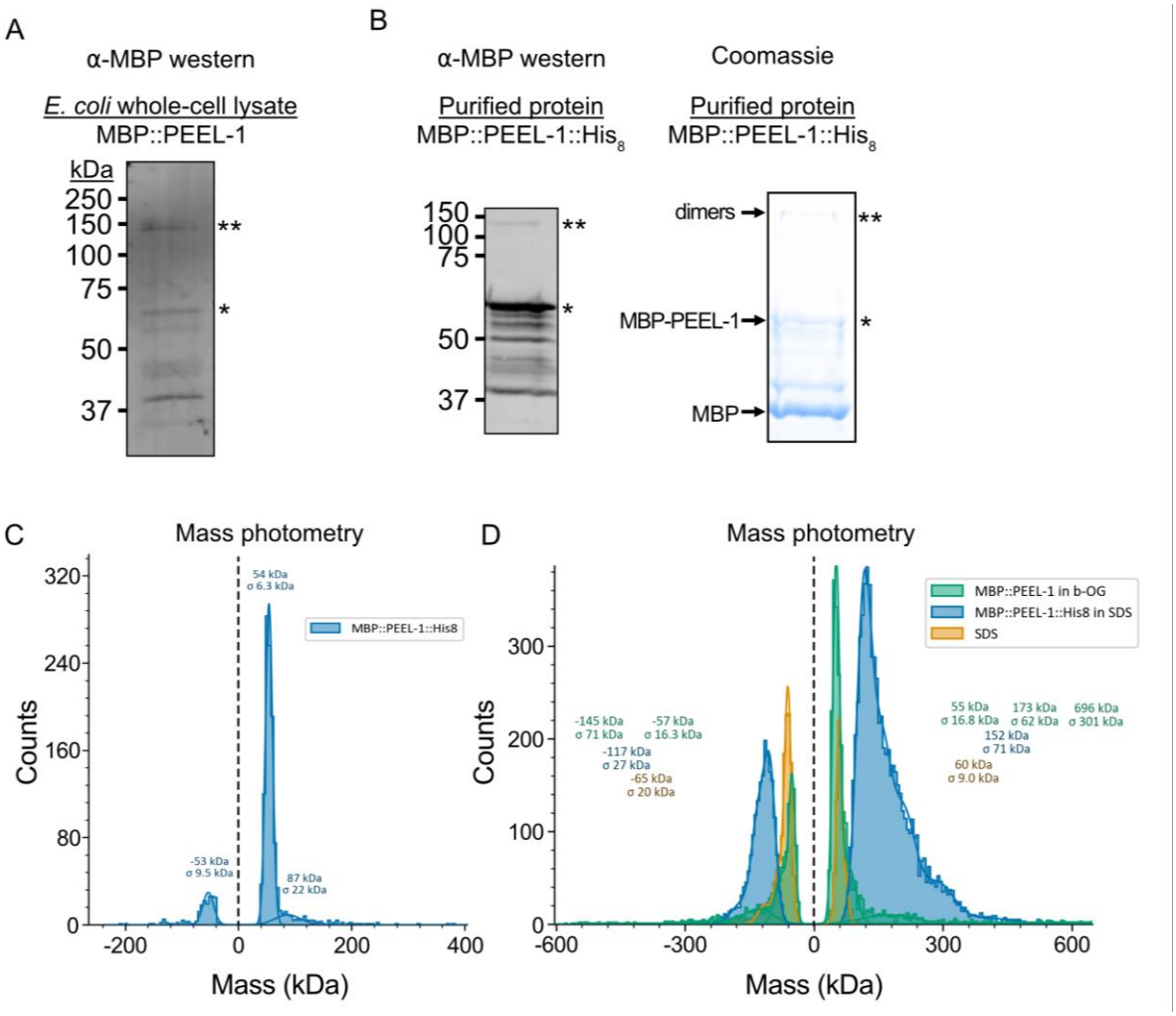

Figure 4.4. PEEL-1 forms SDS-resistant oligomers.

(A-B) SDS-PAGE gels with indicated PEEL-1 samples. Monomers (*) and putative SDS-resistant, dimers (**) are indicated. Monomers of MBP::PEEL-1 are predicted to be 64 kDa. **(A)** Whole-cell lysate anti-MBP western blot of *E. coli* C41(DE3) cells with MBP::PEEL-1 expression induced with 0.5mM IPTG overnight at 18C. **(B)** Western blot (left, anti-MBP) and coomassie stain (right) of purified MBP::PEEL-1::His_8. **(C-D)** Mass photometry of purified MBP::PEEL-1::His_8. Histogram of masses are shown with Gaussian fits. Predicted molecular weight (kDa) and standard deviation (σ) of each peak is shown. Negative masses can be ignored. MBP::PEEL-1::His_8 in beta-octylglucoside is shown in (C). MBP::PEEL-1::His_8 in either beta-

octylglucoside (green) or SDS (blue) is shown in (D). Addition of SDS increases the molecular weight of PEEL-1 protein in solution, possibly through oligomerization or aggregation. Masses of SDS micelles alone, without protein, are also shown (control, yellow).

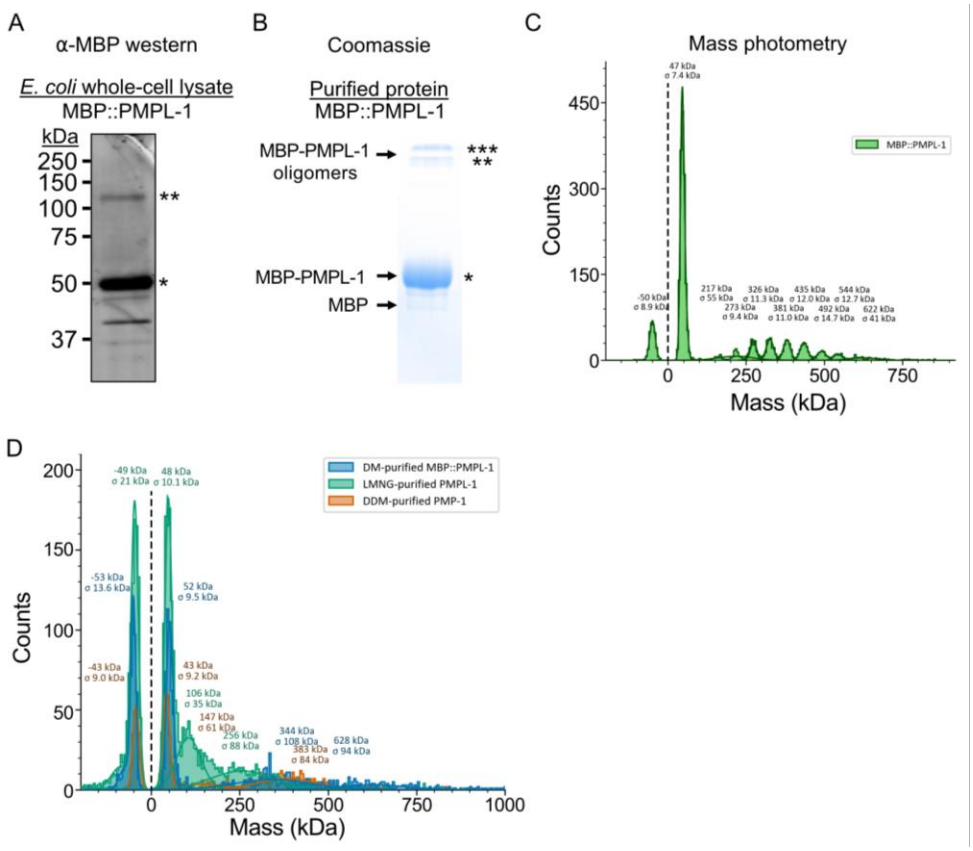

Figure 4.5. PMPL-1 forms SDS-resistant oligomers.

(A-B) SDS-PAGE gels with indicated PMPL-1 samples. Monomers (*) and putative SDS-resistant, dimers (**) and higher-order oligomers (***) are indicated. Monomers of MBP::PMPL-1 are predicted to be 50 kDa. **(A)** Whole-cell lysate anti-MBP western blot of *E. coli* C41(DE3) cells with MBP::PMPL-1 expression induced with 0.5mM IPTG. **(B)** Coomassie stain of purified MBP::PEEL-1. **(C)** Mass photometry of purified MBP::PMPL-1 in beta-octylglucoside (dilution required, resulting in ~0.01% detergent concentration). Histogram of masses are shown with Gaussian fits. Predicted molecular weight and standard deviation of each peak is shown. Negative masses can be ignored. **(D)** Mass photometry of MBP::PMPL-1 purified in n-decyl-beta-maltosome (DM, blue), lauryl maltose neopentyl glycol (LMNG, green), or n-dodecyl-beta-maltoside (DDM, orange).

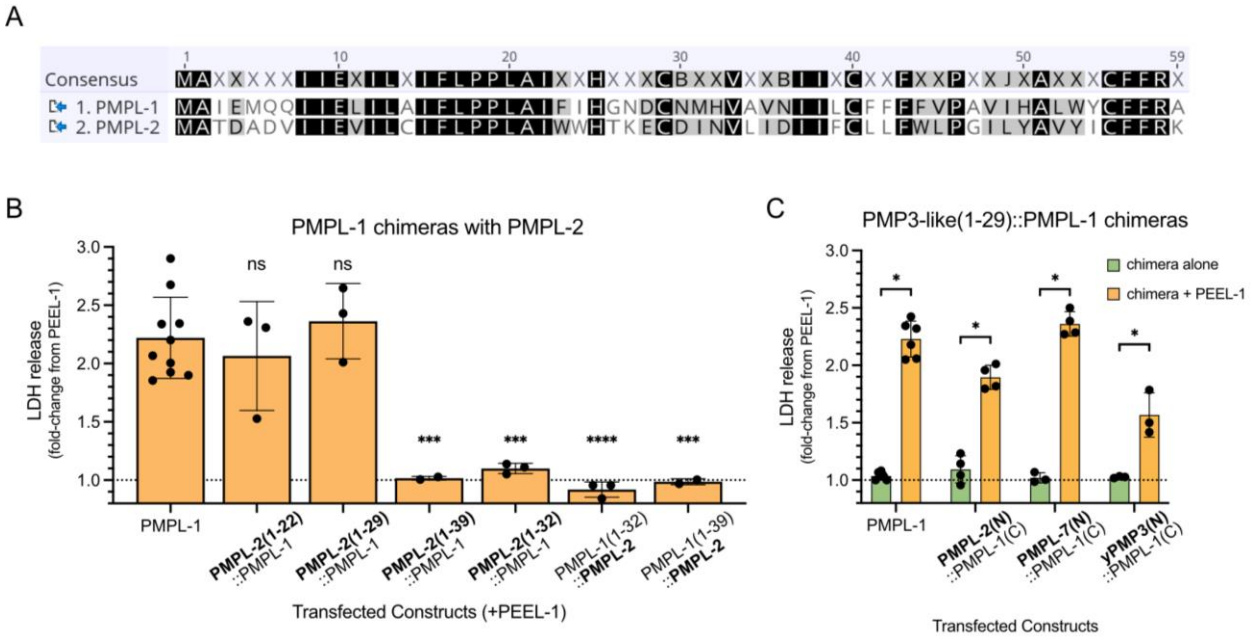

Figure 4.6. Chimeric PMP3-like proteins in HEK293T cells.

(A) Alignment of PMPL-1 and PMPL-2 amino acid sequence from Geneious. Proteins are 47.5% identical and 74.6 % similar. Black boxes indicate identical residues, and gray boxes indicate similar residues. Consensus sequence shown at top. **(B)** LDH assays of PMPL-1 and PMPL-2 chimeras co-transfected with PEEL-1 in HEK293T cells. Individual data points, mean, and SD are shown. Dotted line indicates no killing above PEEL-1 control. Numbers in parentheses corresponds to the amino acids of the indicated PMP3-like protein N-terminus (ex. PMPL-2(1-22)::PMPL-1 indicates a construct coding for the first 22 residues of PMPL-2, fused to residues 23-59 of PMPL-1). **(C)** PMP3-like chimeric constructs transfected alone (green bars) or with PEEL-1 (yellow bars). All chimeras test PMP3-like(N), corresponding to PMPL-1(1-29), fused to PMPL-1(aa30-59) (labeled "PMPL-1(C)"). PMP3-like(N) regions were determined from alignments which match PMPL-1 amino acids 1-29 (ends at a conserved Cys). Statistics in **(B)**: one-way ANOVA with Dunnett's multiple comparisons test. Statistics in **(C)**: multiple unpaired t-tests with Holm-Šídák test, comparing each chimera alone to the same chimera with PEEL-1. PMPL-1 was mCherry tagged or untagged, but all chimeras were untagged. The

yPMP3(N)::PMPL-1 construct had an unintended, two amino-acid insertion in the N-terminus (in-frame, occurred in two independent clones).

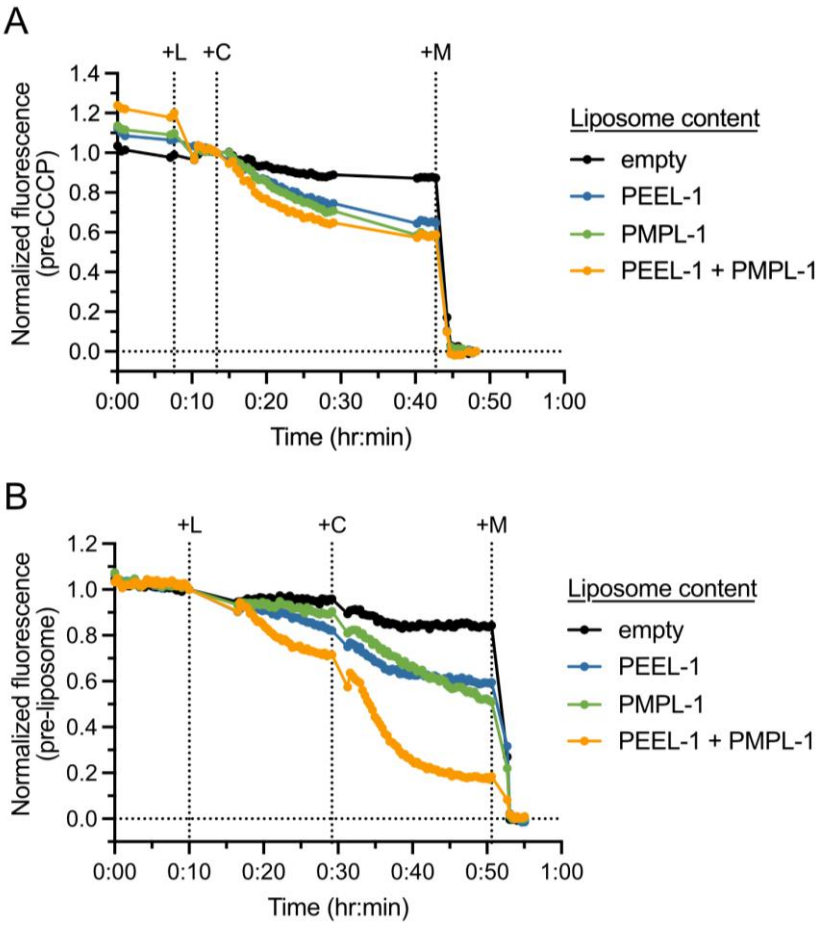

Figure 4.7. Liposome flux assay to measure ion channel activity.

Two examples of the liposome flux assay, measuring fluorescence levels over time in a 96-well plate. Timepoints at which liposomes were added (+L), CCCP (+C), and monensin (+M) are indicated. One fluorescence measurement was recorded every 20 seconds with some gaps occurring due to addition of reagent or instrument error. Normalized fluorescence was calculated by comparing fluorescence after monensin addition to fluorescence before CCCP addition in **(A)** or before liposome addition in **(B)**. The protein content of liposomes are indicated (right). A single experiment is shown in **(A)** and an average of two technical replicates is shown in **(B)**. Technical replicates in (B) were similar.

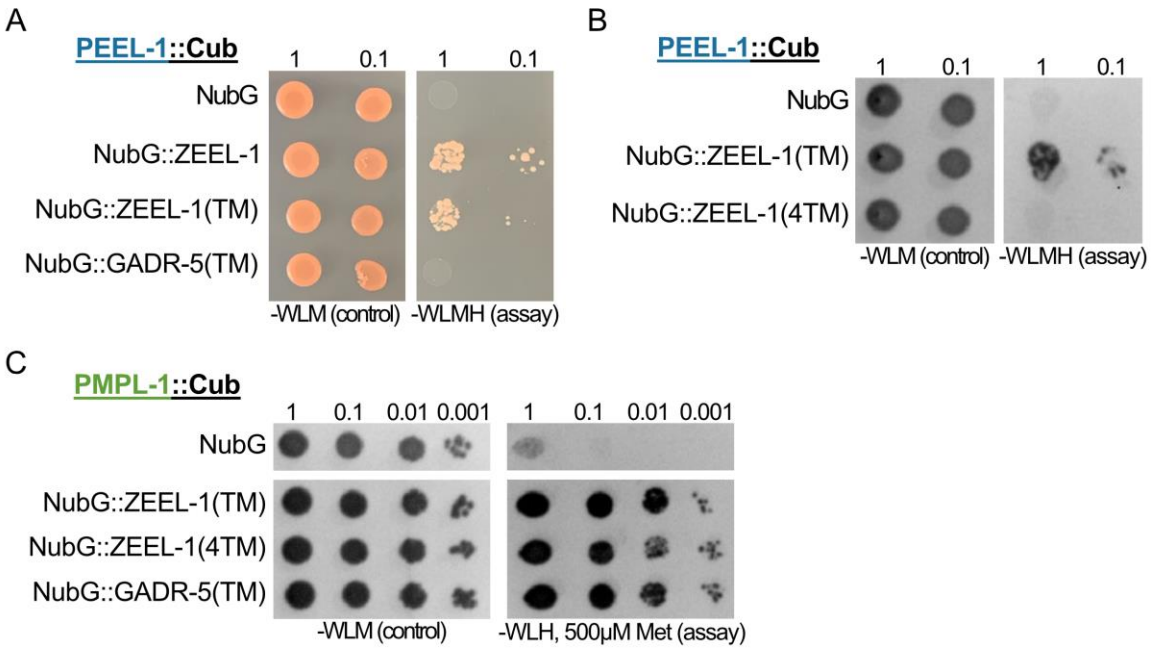

Figure 4.8. mbSUS assaying ZEEL-1 interactions with PEEL-1 and PMPL-1.

(A-B) mbSUS of yeast with PEEL-1::Cub with indicated Nub constructs. NubG is a negative control. Yeast were grown on -WLM media (control, left) or -WLMH media (assay plate, right) for 2 days at 30C. PEEL-1::Cub experiments were repeated at least three times and had similar results. **(A)** PEEL-1::Cub assayed with full-length ZEEL-1, or just the transmembrane domains (TM) of ZEEL-1 or GADR-5 (the closest ZEEL-1 paralog). **(B)** PEEL-1::Cub assayed with a ZEEL-1(4TM) which only contains 4 of the 6 transmembrane domains of ZEEL-1. **(C)** PMPL-1::Cub assayed with indicated constructs. 500µM methionine was used in assay plate to decrease PMPL-1::Cub expression. PMPL-1::Cub experiments were only performed once.

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