Recurrent but Short-Lived Duplications of Centromeric Proteins in Holocentric Caenorhabditis Species

Lews Caro 1,2,† Pravrutha Raman 3,† Florian A Steiner 4, Michael Ailion 1,2 and Harmit S Malik 3,5,*

1Molecular and Cellular Biology Program, University of Washington, Seattle, WA 98195, USA
2Department of Biochemistry, University of Washington, Seattle, WA 98195, USA
3Division of Basic Sciences, Fred Hutchinson Cancer Center, Seattle, WA 98109, USA
4Department of Molecular Biology and Cellular Biology, Section of Biology, Faculty of Sciences, University of Geneva, Geneva, Switzerland
5Howard Hughes Medical Institute, Fred Hutchinson Cancer Center, Seattle, WA 98109, USA

*Corresponding author: E-mail: hsmalik@fhcrc.org
†These authors contributed equally.
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Abstract

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 32 Caenorhabditis species, we find strict retention of the ancestral hcp-3 gene and 10 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 subfunctionalization or functional loss following duplication. An N-terminal extension in the hcp-3L gene of C. africana 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.

Key words: centromeric histone, gene duplication, protein motifs, kinetochore.

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 Facchinetti 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 et al. 2002).
This competition favors changes in centromeric DNA that result in over-recruitment of centromeric proteins (Chmátil 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 (Lemontova et al. 2011; Ravi et al. 2011; Schubert et al. 2014). Conflicting evolutionary selective pressures on CenH3 between these functions (e.g., mitotic vs. meiotic, conserved vs. 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, 2021; Despot-Slade et al. 2021; Elisafenko 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 (HFD) 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; Prosee et al. 2021). This interaction is necessary for the establishment of centromeres in the hermaphroditic germ line, prior to the first embryonic mitosis (Prosee 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; Prosee 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 13 out of 32 analyzed Caenorhabditis species encode two or more CenH3 paralogs, which were the result of at least 10 independent duplication events. We confirm that 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 subfunctionalization. In a possible case of neofunctionalization, we find an unusual CenH3 paralog in C. aafra 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, 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.

Results

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, 1997; Stevens et al. 2019; http://caenorhabditis.org/) to identify all hcp-3 homolog (hcp-3-like) genes (supplementary data S1, Supplementary Material online) and their syntenic location (surrounding genes) (fig. 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 hhl-11 and F58A4.6 (fig. 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, fig. 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 13 out of 32 examined species encode at least one additional hcp-3-like sequence. We refer to these paralogs as “hcp-3L” genes (for hcp-3-like) (fig. 1). These hcp-3L genes include previously reported hcp-3 duplications in C. remanei and C. elegans (Monen et al. 2005, 2015), which we refer to as hcp-3L4 and cpar-1 (as previously named, also referred to as hcp-3L1 in figs. 1 and 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 fig. S1, Supplementary Material online). 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 HFDs (see supplementary data, Supplementary Material online), which are between 69% and 100% identical to the HFD of CHP-3 from the same species (fig. 1). In contrast, their N-terminal domains show high divergence from HCP-3 orthologs (26–97% identical, fig. 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 fig. S2, Supplementary Material online). 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 Caenorhabditis phylogeny. Therefore, we built a maximum-likelihood phylogenetic tree using a codon-based alignment of the conserved HFD cDNA sequence (fig. 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 syntenic 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 (fig. 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 (fig. 2). In each of these seven species, the hcp-3L paralogs are present in unique genomic locations (fig. 1) and typically group most closely with hcp-3 orthologs from the same species (fig. 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. zanzibari. Different genomic locations of hcp-3L duplicates among C. tribulationis, C. sp41, and C. sinica (fig. 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 (fig. 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.

**Fig. 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 gray and labeled 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 HFD).
Thus, it is formally possible that \textit{C. zanzibari} never acquired \textit{hcp-3L2}. However, based on the well-resolved species phylogeny of this quartet of species, we favor the first possibility that \textit{C. zanzibari} acquired, then lost \textit{hcp-3L2}. Therefore, our analyses reveal that \textit{hcp-3} has duplicated at least ten independent times within \textit{Caenorhabditis} species.

We examined the expression of \textit{hcp-3} and \textit{hcp-3L} genes across representative \textit{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 \textit{hcp-3} genes (fig. 3; supplementary fig. S3, Supplementary Material online). We investigated whether
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 (fig. 4A; supplementary fig. S4, supplementary table S3, Supplementary Material), numbered sequentially from the N-terminus, with 11 motifs in the N-terminal tail and motifs 12 and 13 in the 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. beeci (fig. 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 fig. S4, Supplementary Material), 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 (fig. 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
Figure 4. Differential retention of N-terminal tail motifs across HCP-3 and HCP-3L proteins encoded by Caenorhabditis species. (A) Logo plots of 11 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 fig. S4, Supplementary Material) that reside within the HFD. The e-values of all motifs were below 10^{-6}. 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 S4, Supplementary Material). 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 1. A scale bar (number of residues) is shown on the bottom-right.
threshold of the unsupervised motif analysis. Based on these analyses (supplementary data S4, Supplementary Material), we were able to confirm the presence of motifs 1 and 4 in all species (fig. 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 fig. 4A). We predict that mutation of these residues may reveal important insight into 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. tribulatianus 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 acidic segment, ExxR (fig. 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 fig. S5, Supplementary Material). 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 (fig. 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.

Selective Constraints on hcp-3 Orthologs and hcp-3L Paralogs

Our study represents an opportunity to evaluate the selective pressures imposed on ChenH3 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 sub-sets of species from one of the lineages for which we had enough representation (supplementary table S1A, Supplementary Material). 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 (supplementary table S1A, Supplementary Material). 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 ChenH3 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 ChenH3 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 typical 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 (fig. 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 fig. S6, Supplementary Material). We found only three synonymous (amino acid preserving) and zero nonsynonymous (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 nonsynonymous 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 (fig. 4; supplementary fig. S6, Supplementary Material) or HFD, implying that they are likely deleterious for function. In addition to nonsynonymous 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 nonsynonymous 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 (supplementary table S1B, Supplementary Material). 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 (supplementary table S1B, Supplementary Material). 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 (fig. 2). These dS values range from 0.15 (for C. afr a) to 0.74 (for C. doug hertyi) (supplementary table S1B, Supplementary Material). 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 (fig. 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. sp44 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 (fig. 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 (fig. 5A) suggests the possibility of incipient neofunctionalization of the hcp-3L3 paralog in C. sp54.

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. afr a HCP-3 and C. afr a 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. afr a hcp-3L8 contained two copies of C. afr a 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 (fig. 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 (fig. 5C). Therefore, C. afr a hcp-3L8 is a chimera of hcp-4 and hcp-3. In addition to this hcp-4-hcp-3 fusion gene, C. afr a 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. atra, 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 (fig. 6) except for two instances where we were unable to identify full-length intact zwl-1 genes (in C. kamaaina and C. tropicalis) ("*" in fig. 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) (fig. 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 fig. S7, Supplementary Material) like what we observed previously for hcp-3 and hcp-3L genes (supplementary fig. S1, Supplementary Material). 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. atra clades (supplementary table S1C, Supplementary Material). 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 the duplicates are retained under strong purifying selection (supplementary table S1D, Supplementary Material). 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 (fig. 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. afr (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; Proseé 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.

**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, 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
hcpp-3L paralogs we have found are relatively young, assuming that 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 (fig. 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 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 hcpp-3 genes, which would suggest subfunctionalization; 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 hcpp-3L paralogs rather than ancestral hcpp-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. afera, where an HCP-3L protein is fused to an inner kinetochore protein, HCP-4 (CENP-C in mammals; fig. 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 hcpp-3 paralogs in Caenorhabditis species. Nearly half of the species we analyzed contain an hcpp-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 hcpp-3 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 hcpp-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 hcpp-3L paralogs are retained under purifying selection for significant periods of time.

Our evolutionary analyses thus reveal an unusual “revolving-door” of hcpp-3L paralogs in Caenorhabditis species. Under this regime, gene duplication is frequent, hcpp-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 hcpp-3L2 in C. zanzibari), returning to the ancestral state of the genome encoding only a single hcpp-3 gene. This cadence is unprecedented among most other taxonomic groups where CenH3 duplications have been investigated. Even the high number of hcpp-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 hcpp-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, that is, 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 subfunctionalization 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 (supplementary table S1B, Supplementary Material). Similarly, the αCenH3 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 cparr-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 nonmodel organisms and the value of evolutionary comparisons to reveal novelties even in well-studied cellular pathways.

Methods

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).

Strains Used

|   |   |
|---|---|
| N2 | C. elegans |
| DF5081 | C. japonica |
| JU727 | C. sinica |
| JU1333 | C. dougertyi |
| JU2744 | C. tribulationis |
| JU1199 | C. afra |
| VX88 | C. latens |
| QG702 | C. panamensis |

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 32 Caenorhabditis species: C. tribulationis, C. sp41, C. zanizibari, C. sinica, C. nigonii, C. briggsae, C. remanei, C. latens, C. sp51, C. sp44, C. sp48, C. brenneri, C. wallacei, C. tropicalis, C. dougertyi, C. sp54, C. inopinata, C. elegans, C. oii, C. kamaaino, C. waiitukubuli, C. panamensis, C. nouraguensis, C. beeci, C. yunquensis, C. macroperma, C. sulstoni, C. afra, C. sp49, C. sp25, C. imperialis, and C. japonica (supplementary data S2, Supplementary Material). We used tBLASTn (Altschul et al. 1990, 1997) to perform a homology-based search starting with C. elegans HCP-3 (WBGene00001832) 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 (fig. 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 (WBGene0003576), and HIM-10 (WBGene00001869). We used http://blast.caenorhabditis.org/ to perform all tBLASTn analyses using pre-set parameters and setting an 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 neighboring genes as queries for tBLASTn searches of the C. elegans genome to identify the orthologous syntenic genes (fig. 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. The absence of hcp-3 in the ancestral locus in C. sp49 hcp-3 and of hcp-3L2 in the duplicate locus in C. zanizibari was confirmed by using tBLASTn of each gene in the expected locus, resulting in no detectable homologous gene sequence.

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 (five 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, that is, 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 table S4, Supplementary Material presents the pairwise distances and number of differences, respectively, between all hcp-3 and hcp-3L HFD coding sequences.

Motif Analyses

Thirteen 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. owiwi, C. kamaaina, C. waitukubuli, C. nongruensis, C. becei, C. yunquensis, C. macrosperma, C. sulstoni, C. sp25, C. imperialis, and C. japonica). The e-values of all 13 discovered motifs were below $10^{-5}$. Motif logo plots were generated and downloaded from MEME. The 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 the 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 fig. S5, Supplementary Material.

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 HFD (supplementary table S1A, Supplementary Material). 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). To test whether any residues evolve under positive selection, we compared likelihoods between model 8 (where there are 10 classes of codons with dN/dS between 0 and 1, and a 11th class with dN/dS > 1) and model 7 (which disallows codons with dN/dS > 1) or model 8a (where the 11th 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 $\chi^2$ distribution with the degrees of freedom reflecting the difference in the number of parameters between the models being compared (Yang 1997).

C. elegans HCP-3 and CPAR-1 Polymorphisms

To determine natural variation in C. elegans hcp-3 and cp AR-1 genes (supplementary fig. S6, Supplementary Material), 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 nonsynonymous mutations in cp AR-1 were identified by the CeNDR variant annotation feature. The cp AR-1 partial deletion was found manually by looking at whole-genome sequencing reads from C. elegans strain ECA740 mapped onto the N2 reference genome.

RT-PCR

Total RNA was isolated using TRlzol (Fisher Scientific) from 50 to 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 20 µl of nuclease-free water. Next, RNA was treated with DNase I (New England Biolabs, 2 units/µl) at 37°C for 60 min followed by heat inactivation at 75°C for 10 min. 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. afr (in fig. SC), 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 supplementary table S2, Supplementary Material.

Supplementary Material

Supplementary data are available at Molecular Biology and Evolution online.
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Data Availability

The data underlying this article are available in the article and in its online supplementary material. Supplementary data files include sequence alignments used to generate phylogenetic trees and for evolutionary analyses and sequences of all HCP-3 and kinetochore protein orthologs and paralogs described in the paper.

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