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Functional Testing of Human Disease Missense Variants in *Caenorhabditis elegans* by Targeting *COQ2* Variants

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Introduction: Missense variants introduce single amino acid substitutions into proteins that might affect their functions and cause genetic diseases, including kidney disease. The clinical significance of most missense variants in human genes is difficult to determine. We investigated the functional effects of missense variants in highly homologous protein orthologs in humans and the nematode *Caenorhabditis elegans*.

Methods: Ortholog analysis was performed to investigate the utility of *C. elegans* as a model for assessing the functional consequences of human missense variants, particularly those whose clinical significance remains undetermined. By using CRISPR-Cas9 genome editing, we generated *C. elegans coq-2* missense variant mutants that model human missense variants. Phenotypic analyses were conducted to compare pathogenic phenotypes in *C. elegans coq-2* missense variant mutants and those of human primary CoQ10 deficiency.

Results: Approximately 250 ortholog pairs were genes reported to be linked to human genetic diseases and approximately half of documented human variants in these genes were missense variants whose clinical significance remains largely undetermined. We chose to characterize undetermined missense variants in *COQ2*, which encodes coenzyme Q2 polyprenyltransferase (COQ2), as an example to test whether they cause measurable phenotypes when introduced into the orthologous *coq-2* in *C. elegans*. We found marked phenotypes consistent with primary coenzyme Q_{10} (CoQ10) deficiency in humans, and that the phenotypes were generally rescued by CoQ10 supplementation.

Conclusion: Our findings provide insights into human genetic disease–associated missense variants and demonstrate that *C. elegans* can be used as a simple, cost-effective *in vivo* model for testing undetermined missense variants in human disease genes.

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Missense variants are the most prevalent type of genetic change that affects the structure and function of proteins.[1] However, the clinical significance of the vast majority (~98%) of missense variants in the human genome remains undetermined.[2] Therefore, the task of addressing the causal effects of these missense variants and validating pathophysiological consequences is a major challenge.[3]

Among these, mutations in genes encoding enzymes functioning in the biosynthesis of CoQ10 have emerged as critical factors linked to mitochondrial dysfunction and a range of multisystemic disorders, including severe renal pathology.[4,5] COQ2 is a key enzyme in the biosynthesis of CoQ10, and is essential for mitochondrial electron transport chain.[6] Mutations in *COQ2* cause CoQ10 deficiency that causes inherited mitochondriopathy with primary renal involvement, including mitochondrial encephalomyopathy and nephropathy.[7–10] However, assessing the functional consequences of missense variants in COQ2 remains a

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substantial challenge in clinical and experimental settings.

The nematode, *C. elegans* is a major model organism for studying the genetics of biological processes, including aging, development, neurophysiology, metabolism, RNA quality control, and pathogen resistance.[11-19] Despite lacking several anatomical and physiological features observed in humans, such as the brain and adaptive immune systems, *C. elegans* shares 60% to 80% of its genes with human when using broader sequence similarity criteria[20,21] and genetic deletion or silencing of mammalian gene orthologs in *C. elegans* has been widely used to study human genetic diseases.[21,22] For example, *C. elegans* has been used as a kidney research model to elucidate molecular pathways involved in human kidney diseases, despite the lack of apparent excretory system comparable to the mammalian kidney.[20,23,24] *C. elegans* has been used to study the CoQ10 biosynthesis pathway, particularly through mutations in the orthologs of the human genes.[25-29] In addition, transgenic models have been developed to investigate neurodegenerative conditions, such as Parkinson disease, Alzheimer disease, Huntington disease, and amyotrophic lateral sclerosis.[20,21,30] *C. elegans* has been employed to study pathogenicity of human genetic disease variants,[31-38] and in large-scale screens for drugs to treat human genetic diseases.[39] Therefore, *C. elegans* is an excellent model organism to study genetic aspects of human pathophysiology.

In this study, we investigated the possibility of employing *C. elegans* to test whether human missense variants whose clinical significance remains undetermined cause human diseases. By using the 6 ortholog prediction programs, which define orthologs based on sequence similarity, phylogenetic relationships, and ortholog confidence scores, we first obtained a list of $>$ 2200 orthologous gene pairs in humans and *C. elegans*. Among them, we identified 877 genes that encode highly homologous proteins. Approximately 250 of them were genes reported to be linked to human genetic disease and approximately half of 100,000 documented human variants in these genes were missense variants whose clinical significance remains largely undetermined. We chose undetermined missense variants in the *COQ2*, which encodes the enzyme COQ2, as an example to test whether they cause a measurable phenotype when introduced into the orthologous *coq-2* in *C. elegans*, and found marked phenotypes consistent with primary CoQ10 deficiency in humans. Our findings provide insights into human genetic disease–associated missense variants and demonstrate that *C. elegans* can be used as a simple, cost-effective *in vivo* model for testing undetermined missense variants in human disease genes, particularly those implicated in mitochondriopathy associated with primary renal involvement.

METHODS

Generation of Protein Ortholog List Between Human and *C. elegans*

A precompiled list of human and *C. elegans* orthologs (28,298 orthologs) was initially obtained by using OrthoList2[40] (http://ortholist.shaye-lab.org/). Subsequently, 2278 ortholog pairs with score 6 (ortholog pairs identified in 6 of 6 ortholog-prediction programs; InParanoid, OrthoMCL, Ensembl Compara, HomoloGene, OMA, and OrthoInspector) were selected as confident orthologs. The canonical protein sequences of the 2278 orthologs in *C. elegans* were obtained by using UniProt (UniProtKB/Swiss-Prot).[41] The calculation of protein sequence identities and percent positive values between orthologs was performed by using protein-protein BLAST (BLASTP). RefSeq protein database was used for a reference database. BLOSUM62 (scoring parameter) and expect threshold $<$ 1e$-$5 was used for calculating identity values using BLASTP. Overrepresentation analysis of gene ontology (GO) terms in the 252 human and *C. elegans* ortholog pairs reported with human genetic diseases were performed by using WebGestalt[42] based on human GO term annotation. Weighted set cover was applied to reduce the redundancy of enriched terms and to identify 10 representative overrepresented GO terms. Underrepresentation analysis of GO terms in the 252 ortholog pairs reported with human genetic diseases was performed by using g:Profiler[43] based on human GO term annotation.

Analysis of Variants in Genes That are Linked to Genetic Diseases

Among the variants in the genome aggregation database v4.1.0,[44] variants with the Matched Annotation from National Center for Biotechnology Information and EMBL-EBI (MANE) Select transcript were analyzed in this study. The functional effect of variants was annotated using the Variant Effect Predictor and the variants were classified into 2 categories as follows: (i) "exonic variants" that encompasses protein-truncating predicted loss-of-function (pLoF), missense, inframe insertion and deletion, and synonymous variants; and (ii) "other variants" that includes variants located in intronic, untranslated region (UTR), and noncanonical splice regions. The variants reported in the ClinVar were acquired from the ClinVar FTP server (https://ftp.ncbi.nlm.nih.gov/pub/clinvar/tab_delimited/). The "variant_summary.txt.gz" file was

downloaded on February 6, 2024, and were processed by using the following steps: (i) filtration of variants based on the GRCh38 human reference genome; (ii) selection of variants that correspond to canonical transcripts; (iii) classification of variants into 5 annotated clinical significance categories, namely pathogenic/likely pathogenic (P/LP), variants of uncertain significance (VUS), variants for which there are conflicting interpretations of pathogenicity (CIP), variants that are benign or likely benign (B/LB), and all the remaining variants (i.e., Others); and (iv) annotation of variants into 5 types of variants, namely pLoF, missense, inframe insertion and deletion, synonymous, and other intronic or noncanonical splice region/UTR (noncoding) variants. For the analysis of the conservation of amino acid residues of human missense variants in *C. elegans* orthologs, the amino acid sequences of both human and *C. elegans* proteins were retrieved from the National Center for Biotechnology Information database using RefSeq protein accession IDs. The alignment of amino acid sequences between human COQ2 (RefSeq: NP_001345850.1) and *C. elegans* COQ-2 (RefSeq: NP_871684.1) ortholog was performed using the msa R package.[45]

Analysis of COQ2 Protein Ortholog Structure and Sequence

Predicted 3-dimensional structures of human COQ2 (Q96H96) and *C. elegans* COQ-2 (Q8I7J4) were obtained by using AlphaFold2 from UniProt (UniProtKB/Swiss-Prot) in PDB format.[46–48] The 3-dimensional protein structures were aligned by using TM-align.[49] Predicted pathogenicity of COQ2 human amino acid substitutions and missense variants were obtained from the AlphaMissense cloud repository (https://storage.cloud.google.com/dm_alphamissense/AlphaMissense_aa_substitutions.tsv.gz).[2] The prediction of deleterious or pathogenic variants was performed by using PANTHER-PSEP.[50] Protein sequence alignment of human COQ2 and *C. elegans* COQ-2 was performed by using Clustal Omega.[51] Residues were marked from dark blue to white for representing percent identity values by using Jalview.[52] The annotations of UbiA prenyltransferase family domain (Accession: cl00337) in the reference protein sequence of human COQ2 (RefSeq: NP_001345850.1) and *C. elegans* COQ-2 (RefSeq: NP_871684.1) were obtained from the National Center for Biotechnology Information's conserved domain database.[53]

C. elegans Strains and Maintenance

All *C. elegans* strains were maintained at 20 °C on standard nematode growth medium (NGM) plates seeded with OP50 *Escherichia coli* bacteria. *C. elegans* strains used in this study are listed as follows: Bristol strain N2 was used as wild-type; PHX8161 *coq-2 (M69V) III*, PHX8145 *coq-2(T235I) III,* PHX8204 *coq-2 (Y238C) III*, and PHX8430 *coq-2(R114H) III/hT2[bli-4 (e937) let-?(q782) qIs48] (I;III)* were obtained from SunyBiotech (http://www.sunybiotech.com/), which employed CRISPR-Cas9 genome editing. The genotypes of *coq-2* missense variant mutants were confirmed with polymerase chain reaction–based genotyping before assays.

Measurement of Embryonic Lethality

Embryonic lethality was assessed by measuring the hatching rate, as previously described[54] with minor modifications. For each strain, 20 embryos were placed onto new NGM plates seeded with OP50 *E. coli* bacteria and placed in an incubator at 20 °C. After 48 hours, the number of hatched animals was counted to determine the percentage hatching. The assay was conducted 3 times independently. A 2-tailed *t* test was used for statistical analysis. Images indicating embryonic lethality in animals were captured using a digital microscope (DIMIS-M, Siwon Optical Technology, Anyang, South Korea). ImageJ software (v.1.53.) was used to add the scale bar.

Measurement of Swimming

The swimming rate of *C. elegans* was measured as previously described.[55] After synchronization, 10 day-1 adult animals were transferred into a well in a 24-well plate containing 1 ml of M9 buffer. After stabilization of animals in the M9 buffer, the body bending of animals in liquid was recorded using a digital microscope (DIMIS-M, Siwon Optical Technology, Anyang, South Korea). The body bending of individual animals was counted for 30 seconds and converted to the number of bends per minute. Dead animals were excluded from the assays. A 2-tailed *t* test was used for statistical analysis.

Measurement of Pharyngeal Pumping Rates

Pharyngeal pumping (feeding) of *C. elegans* was measured as previously described.[56] After synchronization of each strain, the pumping rate of 10 day-1 adult animals was counted for 30 seconds and then converted to the number of pumps per minute. The rates of pumping were observed under a dissecting microscope (Zeiss SteREO Discovery V8, Zeiss Corporation, Jena, Germany). The assay was conducted 3 times independently. A 2-tailed *t* test was used for statistical analysis.

Measurement of Total Brood Size

Total brood size was measured as previously described[57] with minor modifications. A single L4

stage animal was transferred onto an NGM plate seeded with OP50. Each animal was transferred onto a new NGM plate seeded with OP50 every day until stopping laying eggs for 2 consecutive days. The number of viable larvae descended from a single animal was counted as the brood size. The sum of the progeny numbers for all the counted days was considered as the total brood size. For each strain, brood size was determined by using 15 animals. Animals that died, displayed internal hatching, or crawled off before stopping laying eggs were excluded from the analysis. The assay was conducted 3 times independently. A 2-tailed *t* test was used for statistical analysis.

Measurement of Developmental Time to Adulthood

Developmental time was measured as previously described[58,59] with minor modifications. Hatched L1 larvae were washed off NGM plates using M9 buffer, and the remaining eggs were incubated at 20 °C for 1.5 hours. Newly hatched L1 larvae were transferred onto new NGM plates seeded with OP50 and incubated at 20 °C. After 40 hours of incubation, the number of prefertile adults was counted every 2 hours. The assay was conducted 3 times independently. A 2-tailed *t* test was used for statistical analysis.

Measurement of Defecation Cycle

The defecation cycle was measured as previously described[60] with minor modifications. The time between the contraction of the *C. elegans* rectal muscles and the subsequent excretion to the next excretion was considered as the defecation cycle and was observed under a dissecting microscope (Zeiss SteREO Discovery V8, Zeiss Corporation, Jena, Germany). For each strain, the defecation cycle of 10 day-1 adult animals was counted. The assay was conducted 3 times independently. A 2-tailed *t* test was used for statistical analysis.

CoQ10 Supplementation Assays

CoQ10 supplementation assays were performed as previously described[29] with minor modifications. First, 150 mg CoQ10 (Sigma, St. Louis, MO) was dissolved in 1.5 ml of dimethylsulfoxide (Sigma, St. Louis, MO). This solution was thoroughly mixed with 13.5 ml of 10% Tween 80 (Sigma, St. Louis, MO) to prepare a 10 mg/ml CoQ10 stock. The 15 ml CoQ10 stock was then added to autoclaved NGM at a final concentration of 150 μg/ml. For the control medium, 1.5 ml dimethylsulfoxide was mixed with 13.5 ml of 10% Tween 80 and 15 ml of this solution was added to autoclaved NGM. The assays were conducted on CoQ10-supplemented or control NGM plates starting from the synchronization stage.

Statistical Analysis

The statistical analysis for each *C. elegans* experiment is indicated in Figure legends or the Methods. The threshold for statistical significance was set at 95% significance.

RESULTS

Identification of Protein Orthologs Between Humans and *C. elegans* Associated With Human Genetic Diseases

To investigate whether *C. elegans* can be used to study the causal effects of missense variants in human genes linked to genetic diseases, we looked first for orthologous gene pairs in humans and *C. elegans*. Based on OrthoList2 compendium of *C. elegans* protein-coding genes with human orthologs,[40] we obtained a list of 2278 confident ortholog pairs (Figure 1a and b). Among them, we identified 877 protein pairs with substantial sequence homology,[61] which we considered as *bona fide* protein orthologs (Figure 1a and c, and Supplementary Table S1). These 877 *bona fide* ortholog pairs had sequence identities ≥ 21%, and 821 of them (93.6%) had sequence identities ≥30% (Figure 1c), a cutoff used previously to define orthologs.[62] We observed a strong association between the percent identity and the percent positive value obtained from BLASTP analysis for the 877 ortholog pairs (Figure 1d). In contrast, we observed a negative correlation between the percent identity and the length of the overlapping amino acid sequences (Figure 1e), suggesting that larger proteins are more likely to have diverged than smaller ones. Thus, we assembled a list of 877 highly conserved protein orthologs in human and *C. elegans*.

Of the 877 ortholog pairs, we found that 252 were linked to human genetic diseases reported in the Online Mendelian Inheritance in Man database[63] (Figure 1a and f, and Supplementary Table S1); specifically, these 252 ortholog pairs included 249 human genes and 252 *C. elegans* genes. Among the 877 ortholog pairs, the more highly conserved ortholog pairs (those with ≥ 30% sequence identity) tended to be more likely implicated in human genetic diseases (Figure 1g), whereas the less highly conserved pairs (those with < 30% sequence identity) were less likely to be (Figure 1h). These findings suggest that *C. elegans* mutants with genetic variants in highly conserved protein orthologs might serve as models for investigating the causal effects of human genetic disease variants.

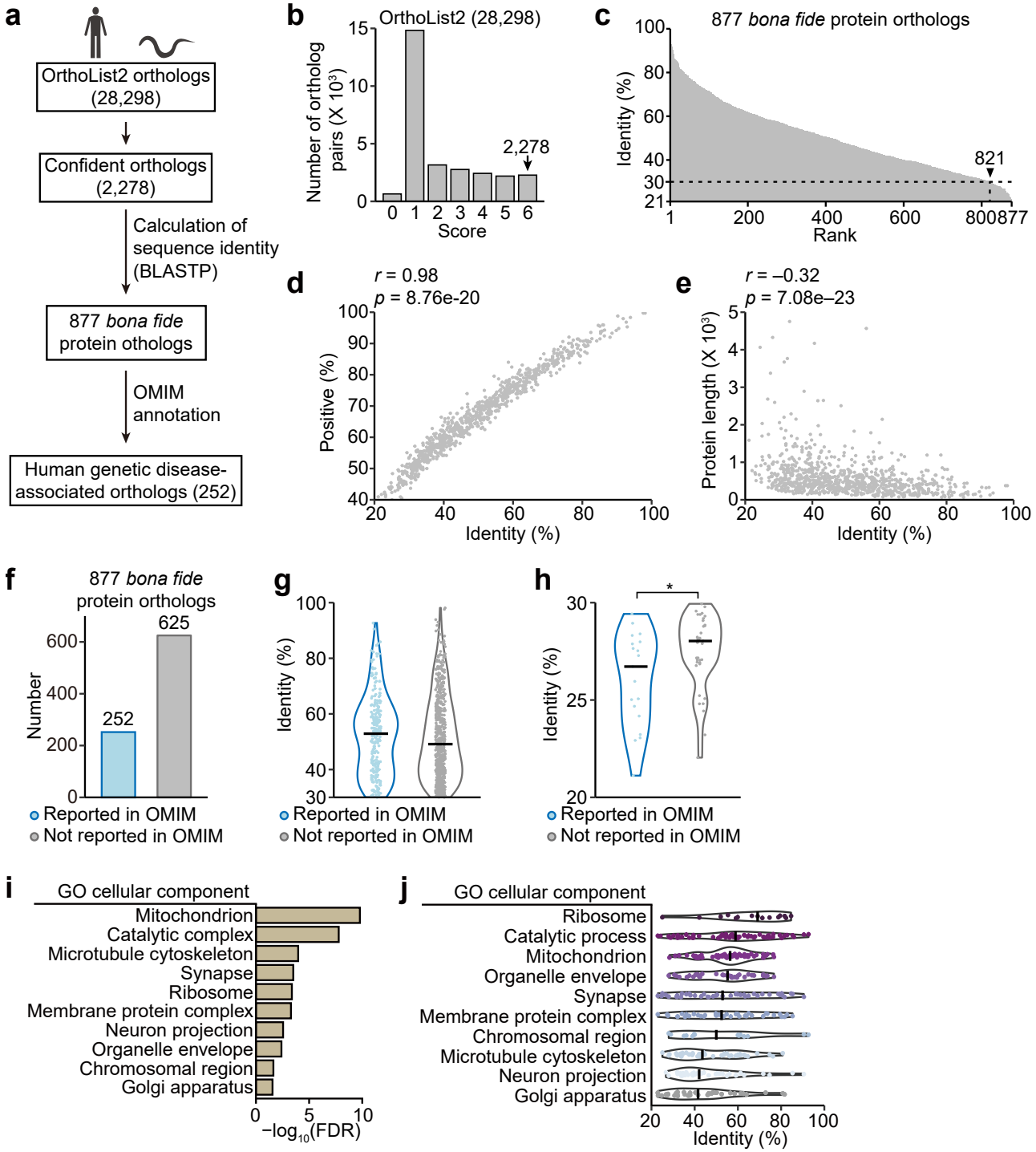

Figure 1. A confident ortholog list between *Caenorhabditis elegans* and human. (a) A schematic diagram showing the strategy for obtaining a confident protein ortholog list between *C. elegans* and human. The ortholog list between *C. elegans* and human was obtained using OrthoList2 (28,298 orthologs). Among them 2,278 orthologs were identified in 6 ortholog-prediction programs. By using BLASTP, 877 pairs displayed substantial sequence homology (BLASTP expect threshold $<$ 1e−5). Among them 252 pairs were reported to be associated with human genetic diseases, based on Online Mendelian Inheritance in Man (OMIM). (b) A summary of the ortholog list in OrthoList2. Scores indicate the numbers of ortholog pairs detected in the 6 ortholog prediction programs. A total of 2278 ortholog pairs were indicated. (c) A density plot of 877 *bona fide* ortholog pairs between human and *C. elegans* calculated by using BLASTP, arranged with percent identity. The *bona fide* 877 orthologs displayed a minimum of 21% sequence identity. Among the 877 orthologs, 821 ortholog pairs exhibited sequence identities higher than 30%. See Supplementary Table S1 for the list of the confident 877 orthologs between *C. elegans* and human. (d) A positive correlation between percent identities and percent positive values between the 877 ortholog pairs. Pearson correlation coefficient *r* and its significance *p* are shown. (e) A negative correlation between percent identities and *C. elegans* protein lengths between the 877 ortholog pairs. Pearson correlation coefficient *r* and its significance *p* are shown. (f) The number of protein ortholog pairs whose variants are reported to be associated with human genetic diseases based on OMIM and whose (continued)

Key Biological Processes are Highly Conserved Between Humans and *C. elegans*

To determine the extent of evolutionary conservation of biological function regulated by the 252 human and *C. elegans* ortholog pairs reported with human genetic disease, we compared their GO term enrichments by performing overrepresentation analysis. The 252 orthologs were analyzed for 3 different GO categories based on human GO term annotation: cellular component (Figure 1i and j), biological process (Supplementary Figure S1a and b), and molecular function (Supplementary Figure S1c and d). We applied weighted set cover to reduce the redundancy of terms and to identify the 10 most represented terms in each category. We then examined the 10 significantly enriched terms by statistical significance (Figure 1i, Supplementary Figure S1a and c) and then by percent identity of protein ortholog pairs in each term (Figure 1j, Supplementary Figure S1b and d). The 10 most significantly enriched GO cellular component terms were the following: mitochondrion, catalytic complex, microtubule cytoskeleton, synapse, ribosome, membrane protein complex, neuron projection, organelle envelope, chromosomal region, and Golgi apparatus (Figure 1i and j). The 10 most significantly enriched GO biological process terms were the following: small molecule metabolic process, organonitrogen compound biosynthetic process, generation of precursor metabolites and energy, organonitrogen compound catabolic process, organophosphate metabolic process, response to organic cyclic compound, protein-containing complex assembly, central nervous system development, establishment of localization in cell, and cellular response to stress (Supplementary Figure S1a and b). The 10 most significantly enriched GO molecular function terms were the following: purine nucleotide binding, oxidoreductase activity, vitamin binding, protein-containing complex binding, pyrophosphatase activity, ubiquitin protein ligase binding, protein dimerization activity, lyase activity, RNA binding, and acyltransferase activity (Supplementary Figure S1c and d). In contrast to these conserved processes, GO terms that are underrepresented appear to be involved in diverged processes, including adaptive immunity (e.g. humoral immune response, alpha-beta T cell activation, cytokine activity, and antigen binding; Supplementary Figure S1e–g). These data indicate that the proteins responsible for essential biological processes in eukaryotes tend to be conserved between humans and *C. elegans*.

Missense Variants in Conserved Residues are Most Likely to be Associated With Human Genetic Disease

We used the genome aggregation database, which collates and analyzes human genetic variation in whole genome or exome sequencing data of 807,162 individuals,[44] to analyze genetic variants in the 249 human genes of the 252 ortholog pairs reported to be linked to human genetic diseases. We found 884,440 variants in the 249 genes, 40% of which were in exons (Figure 2a, Supplementary Table S2), and 60% in introns, noncanonical splice regions, or UTRs (Figure 2a, and Supplementary Table S2). Of the 884,440 variants, only 8.4% (73,823 variants) were reported in the ClinVar database of reports of human variations classified for diseases[64] (Figure 2b), indicating that a large fraction of the variants lacks functional annotation.

In the ClinVar database, we identified 101,097 genetic variants in the 249 human genes associated with genetic diseases, which we classified into 5 categories based on their annotations of clinical significance as follows: P/LP, CIP, VUS, B/LB, and all the remaining variants (i.e., Others) (Figure 2c and d, and Supplementary Table S3). In addition, we classified the variants into categories based on their annotations of types of variants: pLoF, missense, inframe insertion and deletion, synonymous, and other intronic/noncanonical splice region/UTR (noncoding) variants (Figure 2e and Supplementary Table S3). By comparing the clinical significance annotations with the types of each variant, we found that $>$ 50% of P/LP variants were also pLoF variants, whereas most B/LB variants were either synonymous or other noncoding variants (Figure 2f and Supplementary Table S4a). Variants classified as Others for their clinical significance included pLoF, missense, and other noncoding variants (Figure 2f and Supplementary Table S4a). Importantly, $>$ 50% of VUS or CIP variants were missense variants (Figure 2f), whose consequences for human pathology are notoriously difficult to ascertain.

Figure 1. (continued) genetic disease association are not reported in OMIM (Not reported in OMIM). (g,h) Association of OMIM-based reported human genetic diseases in protein orthologs with (g) high ($\geq$ 30%) and (h) low ($<$ 30%) sequence identities. *$P < 0.05$, 2-tailed Wilcoxon rank sum exact test. (i and j) The 10 representative gene ontology (GO) cellular component terms enriched in 252 protein orthologs between human and *C. elegans* reported with human genetic diseases, arranged by (i) $-\log_{10}$(FDR) values and (j) by median values of percent identity. Overrepresentation of GO terms was analyzed by using WebGestalt.[42] Weighted set cover was applied to reduce the redundancy of terms and to identify the ten representative GO terms. FDR: false discovery rate.

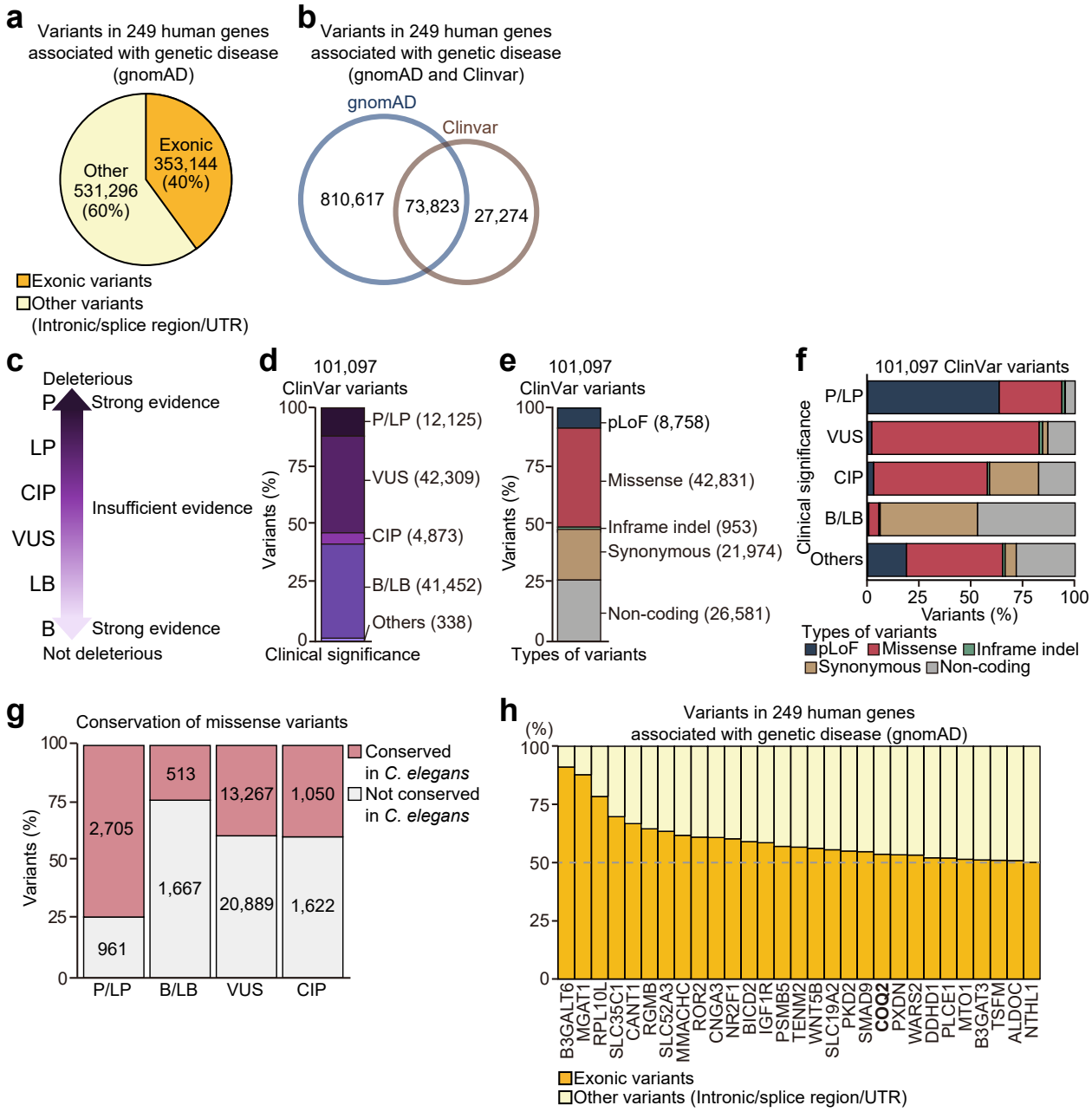

Figure 2. Missense variants at evolutionarily conserved residues are more likely to be pathogenic. (a) 884,440 variants in 249 disease-associated human genes reported in the gnomAD. Among the 884,440 variants, 40% were exonic variants (exonic) and 60% were variants located in intronic, noncanonical splice regions, and untranslated region (UTR) (Other). Supplementary Table S2 contains the summary of variants in 252 orthologs pairs associated with human genetic diseases between *Caenorhabditis elegans* and human. (b) Venn diagram showing variants reported in the gnomAD and ClinVar. (c) Simplified diagram of the clinical significance annotation categories; pathogenic/likely pathogenic (P/LP), conflicting interpretations of pathogenicity (CIP), variants with uncertain significance (VUS), and benign/likely benign (B/LB). (d and e) The 5 (d) clinical significance annotation (clinical significance) categories and (e) types of variants of total 101,097 variants in 249 disease-associated human genes reported in the ClinVar. Five types of variants; predicted loss-of-function (pLoF), missense, inframe insertion and deletion (inframe indel), synonymous, and other intronic/noncanonical splice region/UTR (noncoding) variants. Supplementary Table S3 contains the list of annotated genetic variants that occurred in 249 disease-associated human genes reported in ClinVar database. (f) Percent distribution of 5 different types of variants within each of the 5 clinical significance annotation categories. Supplementary Table S4a includes the summary of ClinVar variants classified into 5 categories based on clinical significance annotation and types of variants. (g) Clinical significance annotation of evolutionarily conserved missense variants between humans and *C. elegans*. (h) A total of 29 of 249 disease-associated human genes which had a higher proportion of exonic variants than variants in intronic, noncanonical splice regions, and UTRs, arranged in the order of exonic variant proportion based on the gnomAD. The pathogenicity of *COQ2* variants (shown in bold) was functionally tested using *C. elegans coq-2* missense variant mutants (Figures 3 and 4). gnomAD: The Genome Aggregation Database.

To identify human missense variants that might cause disease-like phenotypes in *C. elegans*, we looked for variants at residues that are conserved in the human and *C. elegans* orthologs. Among 42,831 missense variants of the 249 disease-associated human genes in the ClinVar database, 3666 were P/LP; 2180 were B/LB; 34,156 were VUS, and 2672 were CIP variants (Figure 2g). Of these P/LP missense variants, 2705 (73.8%) occurred at conserved residues in *C. elegans* (Figure 2g). In contrast, only 513 B/LB variants (23.5%) occurred at conserved residues in *C. elegans*. These data suggest that variants that have severer consequences for human pathologies are more conserved in *C. elegans* than those that have milder ones. Notably, 36,828 of 42,831 missense variants (85.9%) were classified as VUS or CIP (Figure 2e and g). Thus, the pathological effects of the vast majority of missense variants in the 249 human genes associated with genetic diseases can be tested in *C. elegans*.

Among the 34,156 missense variants classified as VUS, 13,267 (38.8%) occurred at residues that were evolutionarily conserved in their *C. elegans* orthologs (Figure 2g). In addition, 39.3% (1050/2672) missense variants classified as CIP occurred at residues that were conserved in *C. elegans* (Figure 2g). These missense variants in conserved residues of orthologs are candidates for phenotypic testing in *C. elegans*.

VUS and CIP variants pose difficulties in the field of molecular genetic diagnostics. Therefore, we sought to obtain functional information that predicts the effects of VUS and CIP variants on genetic diseases, by using the ortholog pairs between human and *C. elegans*. As an approach to select a subset of candidate genes whose VUS or CIP missense variants might be tested in *C. elegans*, we arranged the 249 human genes associated with genetic diseases in an order, according to the proportion of their variants in exons, as reported in the genome aggregation database. We found that 29 of the 249 genes had more variants in exons than in introns (Figure 2h and Supplementary Figure S2a). Notably, $>$ 40% of variants in these 29 genes were classified clinically as VUS and CIP (Supplementary Figure S2b). As a proof-of-concept, we further analyzed VUS or CIP missense variants in the 29 disease-associated human genes for phenotypic testing in *C. elegans*; all the VUS and CIP missense variants of the 249 genes are worth testing in future research.

COQ2 Encodes a Highly Conserved Protein Whose Variants are Associated With Human Disease

To test whether the effects of undetermined VUS and CIP missense variants of a human genetic disease gene might be observed in *C. elegans*, we studied the *COQ2* gene, the ortholog of *coq-2* in *C. elegans*. This gene encodes the enzyme COQ2, a mitochondrial inner membrane protein that is crucial for CoQ10 (or ubiquinone) biosynthesis. Aberrant CoQ10 biosynthesis causes defects in electron transport and oxidative phosphorylation.[65] Mutations in *COQ2* are a frequent cause of primary CoQ10 deficiency and are associated with a broad spectrum of conditions, including encephalomyopathy, nephropathy, and myopathy in humans (Online Mendelian Inheritance in Man entry number 607426).[4,66] We chose *COQ2* for several reasons. First, it was included in the most significantly enriched GO cellular component term, mitochondrion (Figure 1i), associated with genes whose variants are implicated in human genetic diseases.[67] Second, the *COQ2*/*coq-2* orthologs encode protein sequences that are $>$ 57% identical and were among the top third of the 877 ortholog pairs (Supplementary Figure S2c). Third, $>$ 40% of COQ2 variants were VUS or CIP variants (Supplementary Figure S2b).

Alignment of the amino acid sequences of the COQ2 orthologs showed that they are highly conserved among human, mouse, zebrafish, fruit fly, and *C. elegans*, particularly in the UbiA prenyltransferase domain (Figure 3a and Supplementary Figure S2D). In addition, the predicted 3-dimensional protein structures of human COQ2 and *C. elegans* COQ-2 obtained by using AlphaFold2 were very similar, as indicated by a template-modeling score of 0.85 (where a score of 1 indicates perfect structural alignment[49] (Figure 3b and Supplementary Figure S2e and f).

We analyzed human *COQ2* gene variation in the genome aggregation database and disease-associated *COQ2* gene variants in the ClinVar database. The *COQ2* gene was among those in which there was a greater proportion of variants in exons than in introns, noncanonical splice regions, or UTRs (Figure 2h and Supplementary Figure S2a). Of the 272 *COQ2* variants analyzed, $>$ 45% were categorized clinically as VUS and CIP variants (Figure 3c and Supplementary Table S4b, Supplementary Figure S2b), and 43% were missense variants (Figure 3d and Supplementary Table S4b). Moreover, $>$ 50% of the 272 *COQ2* variants categorized clinically as VUS or CIP were missense variants (Figure 3e and Supplementary Table S4b).

For each residue in human COQ2 and *C. elegans* COQ-2, we calculated the probability that a missense variant would have a deleterious effect (Pdel) by using the PANTHER-PSEP,[50] which predicts nonsynonymous genetic variants that may play a role in human disease. This analysis predicted that missense variants in the UbiA prenyltransferase domain were most likely to cause deleterious effects compared with

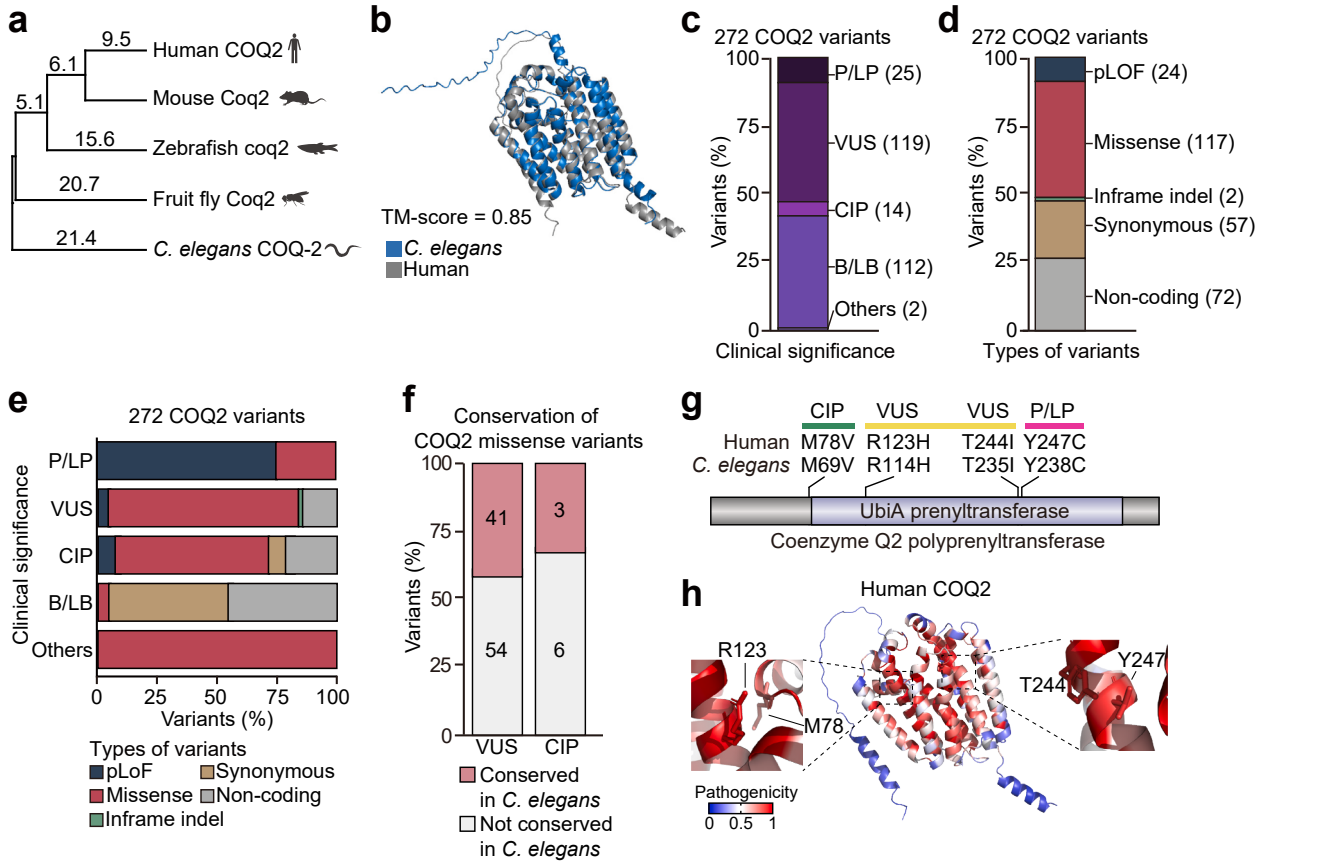

Figure 3. Coenzyme Q2 polyprenyltransferase is highly conserved between human and *Caenorhabditis elegans*. (a) COQ2 orthologs are conserved in the 5 analyzed species. The phylogenetic tree was constructed by using an average distance method based on percent identity. (b) Overlap of 3-dimensional structures of COQ2 orthologs obtained by using PyMOL. Template modeling (TM)-score calculated by using structural alignment tool TM-align was 0.85. (c and d) (c) Clinical significance annotation and (d) types of variants categories of 272 *COQ2* variants reported in the ClinVar. (e) Percent distribution of 5 types of variants within each clinical significance annotation category for *COQ2* in the ClinVar. Supplementary Table S4b includes the summary of *COQ2* variants classified into 5 categories based on clinical significance annotation and types of variants. (f) Evolutionary conservation of 95 missense VUS and 9 missense CIP variants in COQ2. Supplementary Table S5 contains the list of 44 *COQ2* VUS or CIP missense variants conserved in *C. elegans*. (g) Four *C. elegans coq-2* missense variant mutants with substitutions at evolutionarily conserved residues as follows: 1 CIP (green) M69V (human M78V), 2 VUS (yellow) R114H (human R123H) and T235I (human T244I), and 1 P/LP (pink) Y238C (human Y247C). (h) Human *COQ2* missense variants at M78, R123, T244, and Y247 are predicted to be pathogenic by using AlphaMissense. The average pathogenicity of missense variants in human *COQ2* was calculated by using AlphaMissense. Missense variants in UbiA polyprenyltransferase domain were predicted to be more pathogenic (red) than those in the other regions (white and blue).

variants in other domains (Supplementary Figure S3a and b).

Among 95 *COQ2* variants categorized as VUS and missense, 41 were located in residues conserved in *C. elegans*, and among 9 *COQ2* variants categorized as CIP and missense, 3 were located in conserved residues (Figure 3f and Supplementary Table S5).

Most of these VUS and CIP variants located in conserved residues were mapped to the UbiA prenyltransferase domain (Supplementary Figure S3c). Representative conserved CIP and VUS *C. elegans coq-2* variants include 1 CIP variant, *coq-2(M69V)* [equivalent to human *COQ2(M78V)*]; and 2 of the 41 VUS variants, *coq-2(R114H)* [equivalent to human *COQ2 (R123H)*] and *coq-2(T235I)* [equivalent to human *COQ2(T244I)*] (Figure 3g). The 3 aforementioned *coq-2* variants were predicted to be pathogenic in humans by using AlphaMissense (Figure 3h) and in *C. elegans* by using PANTHER-PSEP (Supplementary Figure S3b), similar to *coq-2(Y238C)* [equivalent to human *COQ2(Y247C)*] variant which was previously reported as P/LP variant (Figure 3g and h, and Supplementary Figure S3a–d).

Together, these analyses suggest that missense variants in human *COQ2*, particularly missense variants of undetermined clinical significance, provide an adequate example to test our hypothesis that human diseases caused by missense variants can be modeled in *C. elegans*.

Missense Variants in Evolutionarily Conserved Residues in COQ-2 Cause Pathophysiological Phenotypes in *C. elegans*

To determine whether the pathogenic phenotypes observed in patients with *COQ2* variants might be recapitulated in *C. elegans*, we used CRISPR-Cas9 genome editing to generate the 4 previously described variant mutants in *C. elegans*; 1 CIP variant, *coq-2 (M69V)* [equivalent to human *COQ2(M78V)*]; 2 VUS variants, *coq-2(R114H)* [equivalent to human *COQ2 (R123H)*] and *coq-2(T235I)* [equivalent to human *COQ2 (T244I)*]; and 1 P/LP variant, *coq-2(Y238C)* [equivalent to human *COQ2(Y247C)*] (Figure 3g and h, and Supplementary Table S6). *C. elegans* with defective mitochondria exhibit pleiotropic and pathogenic phenotypes, including slow development, motility, feeding, defecation cycles, and reduced brood size.[68] We therefore measured these phenotypic parameters in the 4 *C. elegans coq-2* missense variant strains. Homozygous *coq-2(R114H)* variants caused semi-embryonic lethality, indicating the severe pathogenic nature of this allele, whereas *coq-2(Y238C)*, *coq-2(M69V)*, and *coq-2 (T235I)* variant mutants were viable (Figure 4a and b). All 3 viable strains, *coq-2(Y238C)*, *coq-2(M69V)*, and *coq-2(T235I)*, exhibited slower swimming (Figure 4c) and pharyngeal pumping (feeding) rates (Figure 4d) compared with the wild-type animals, and these reduced fitness traits were recovered by CoQ10 supplementation. These results suggest that *coq-2* missense variants cause mitochondrial dysfunction, resulting in motility defects similar to myopathies observed in multiple tissues of human CoQ10-deficient patients.[66] Interestingly, CoQ10 supplementation increased swimming and pharyngeal pumping rates in wild-type animals, suggesting that CoQ10 enhances fitness, consistent with its reported longevity-promoting effects.[29] The *coq-2(Y238C)*, *coq-2(M69V)*, and *coq-2(T235I)* variant mutants displayed reduced brood sizes, which were recovered by CoQ10 supplementation (Figure 4e). In addition, all 3 viable *coq-2* variant mutants exhibited delayed development to adulthood, which was ameliorated by CoQ10 supplementation (Figure 4f). *coq-2 (Y238C)*, *coq-2(M69V)*, and *coq-2(T235I)* variant mutants displayed delayed defecation cycle (Figure 4g); however, unexpectedly, CoQ10 further prolonged the defecation cycle, possibly due to its poor solubility[69] (Figure 4g). These varying pathogenic phenotypes caused by *coq-2* missense variants are consistent with variable severity of primary CoQ10 deficiency in humans[66] (Supplementary Table S7). Overall, our findings demonstrate that human missense variants of undetermined clinical significance can cause phenotypes in *C. elegans* that may be used to infer the clinical significance of missense variants in human disease.

DISCUSSION

In this study, we set out to investigate the possibility that *C. elegans* could be used to test whether human missense variants whose clinical significance remains undetermined might cause human diseases. We demonstrate that *C. elegans* provides an effective model for testing the function of missense variants in evolutionarily conserved orthologs. Orthologs of genes whose variants are implied in human diseases are enriched with GO terms related to evolutionarily conserved cellular components, biological processes, and molecular functions. By analyzing databases of human genetic diseases and genetic variation, we conclude that over half of genetic variants whose clinical significance is undetermined (VUS and CIP variants) are due to missense variants. Over 30% of these VUS and CIP missense variants occur in residues that are evolutionarily conserved between human and *C. elegans*. We focused on testing the pathogenicity of undetermined missense variants in *COQ2*, which encodes COQ2 in the CoQ10 biosynthetic pathway.[6] When introduced into the *C. elegans coq-2* gene, missense variants in conserved residues in human COQ2 caused phenotypes consistent with the pathogenic features of loss of COQ2 function in humans, and CoQ10 supplementation ameliorated these features. Two of these *COQ2* variants, which were previously classified as VUS or CIP variants [*COQ2(M78V)* and *COQ2(T244I)*] can now be classified as P/LP variants. Our findings suggest that *C. elegans* provides an invaluable model for categorizing the pathogenicity of tens of thousands of human missense variants in around 250 disease-related genes.

Primary CoQ10 deficiency is caused by pathogenic variants in genes involved in the CoQ10 biosynthetic pathway, including *COQ2*. CoQ10 plays an essential role in the mitochondrial respiratory chain and protects against damage from excessive reactive oxygen species.[10,70] Primary CoQ10 deficiency is associated with severe renal pathologies, including steroid-resistant nephrotic syndrome, collapsing glomerulopathy, and tubular interstitial diseases.[7,10,71,72] Mutations in genes crucial for CoQ10 biosynthetic pathway, such as *COQ2*, *COQ6*, *COQ8*, and *COQ9*, have been reported to cause renal pathologies.[7,10,72,73] Although the mitochondrial disorder caused by primary CoQ10 deficiency can be treated by supplementation with CoQ10,[4,63,74,75] the efficacy of CoQ10 supplementation depends on the specific gene affected and the variant that the patient possesses. The lack of comprehensive genotype-phenotype studies to determine which variants can be effectively treated with CoQ10 supplementation makes it challenging to devise therapeutic

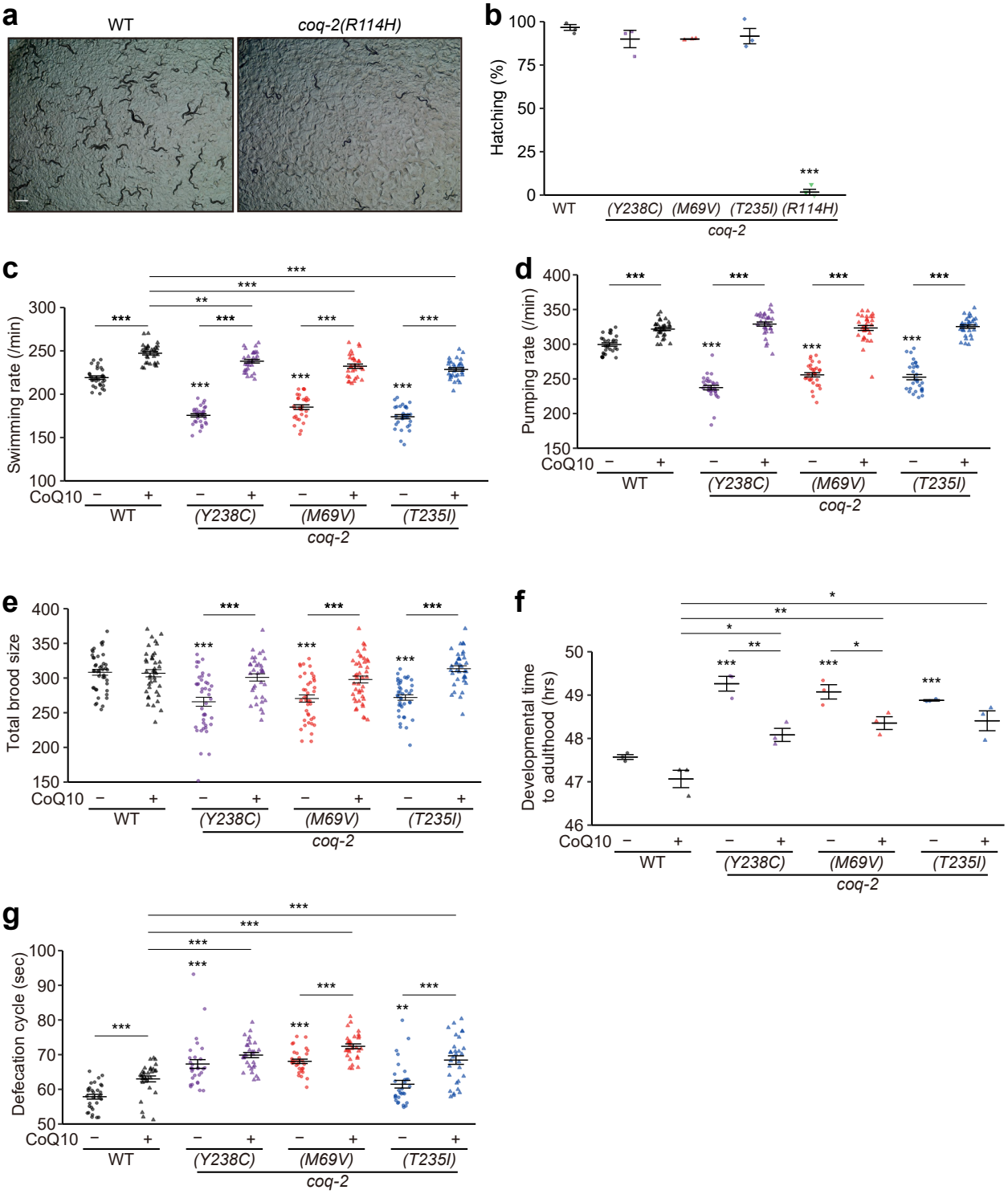

Figure 4. Missense variants in *coq-2* cause physiological defects in *Caenorhabditis elegans*, similar to human genetic disease patients. (a) Images showing wild-type (WT) animals that proliferate and *coq-2(R114H)* variant mutants with a semiembryonic lethal phenotype. Scale bar: 500 μm. (b) Percentage of WT, *coq-2(Y238C)*, *coq-2(M69V)*, *coq-2(T235I)*, and *coq-2(R114H)* variant mutant larvae that hatched ($n = 60$ for each condition from 3 independent trials). (c–g) (c) Swimming rates ($n = 30$ for each condition from 3 independent trials), (d) pharyngeal pumping (feeding) rates ($n = 30$ for each condition from 3 independent trials), (e) total brood size ($n = 43$, 41, 40, and 40 for the control groups of WT, *coq-2(Y238C)*, *coq-2(M69V)*, and *coq-2(T235I)*, respectively; and $n = 39$, 34, 43, and 36 for the CoQ10-supplemented groups of WT, *coq-2(Y238C)*, *coq-2(M69V)*, and *coq-2(T235I)*, respectively, from 3 independent trials), (f) developmental time to adulthood ($n = 133$, 171, 127, and 143 for the control groups of WT, *coq-2(Y238C)*, *coq-2(M69V)*, and *coq-2(T235I)*, respectively; and $n = 115$, 129, 140, and 160 for the CoQ10-supplemented groups of WT, *coq-2(Y238C)*, *coq-2(M69V)*, and *coq-2(T235I)*, respectively, from 3 independent trials), and (g) defecation cycle ($n = 30$ for each condition from 3 independent trials) of WT, *coq-2 (Y238C)*, *coq-2(M69V)*, and *coq-2(T235I)* variant mutants. While measuring defecation cycles with CoQ10 supplementation, we observed black pigments accumulated at the rectal regions, possibly due to the insolubility of CoQ10. We speculate that this black (continued)

approaches tailored to individual patients. Our study showing the usefulness of genetically modified *C. elegans* for recapitulating phenotypes of variants identified in humans will be invaluable as a fast and cost-effective strategy in future medical research, particularly when immediate treatments applicable to patients are unavailable. Integration of functional *C. elegans* data into a database or resource will support timely pathogenicity interpretation and guide treatment strategies for patients with specific variants.

Many mutations in mitochondrial respiration genes are associated with human genetic diseases and cause pleiotropic defects in *C. elegans*. For example, mutations in the *C. elegans* 5-demethoxyubiquinone hydroxylase gene *clk-1* (*COQ7* in humans) and in the NADH-ubiquinone oxidoreductase gene *nuo-1* (*NDUFV1* in humans) reduce health parameters in *C. elegans* and cause mitochondria-associated genetic diseases in humans, including cardiomyopathy, gastrointestinal obstruction, and muscular dystrophy.[76-78] The phenotypes we observed in *coq-2* mutants with missense variants at evolutionarily conserved residues are also commonly observed in the aforementioned mitochondrial mutants. This implies that missense variants in other human genes associated with impaired mitochondrial function might be tested using *C. elegans* in future studies. In addition, direct observation of phenotypes at the organelle and/or molecular level, including mitochondrial function and morphology, will provide valuable complementary insights into the pathogenicity of mitochondrial missense variants.

In addition to mitochondrial proteins, other highly conserved orthologs can be tested for the pathogenic effects of missense variants on human genetic diseases. For example, our GO analysis indicated that ribosomal protein components are highly conserved between human and *C. elegans* (Figure 1i and j). Thus, the potential of missense variants to cause ribosomopathies[79] can be tested in *C. elegans* by altering the sequences of conserved ribosomal protein-encoding genes. In addition, it would be interesting to test whether the pathogenicity of human missense variants in other highly conserved proteins responsible for crucial biological processes, such as synaptic transmission, chromatin regulation, and trafficking through Golgi apparatus, is recapitulated in *C. elegans*.

C. elegans is an excellent model in which to test human genetic diseases. It is suitable for large population analysis because of its short life cycle and fecundity.[80] Experiments using *C. elegans* can be performed in a relatively short time compared with mammalian genetic models, such as mice and rats. *C. elegans* is genetically tractable to easily identify genes by using RNAi screening, random mutagenesis, and CRISPR-based genome editing.[81-84] In addition, many strains and genetic variants are available from repositories, such as those generated by the Million Mutation Project[85] deposited at the *Caenorhabditis* Genetics Center. Whereas mammalian cell culture systems can be useful to study the pathogenicity of genetic variants at the cellular level, the effects of the variants on organism physiology cannot be easily tested. As a metazoan, *C. elegans* is a versatile physiological model with a wide range of molecular and genetic tools,[22,86] suitable for studying the effects of variants both at the cellular and the organismal levels. For example, numerous studies have demonstrated that ciliary dysfunction, ciliopathies, and nephrology can be effectively analyzed by using *C. elegans* as a model organism, particularly by targeting conserved protein orthologs involved in the human kidney filtration barrier.[23,24] Overall, our study suggests that *C. elegans*, an underexploited but cost-efficient multicellular genetic model animal, has the potential to offer key clues to the functional classification of undetermined variants of human disease genes, including kidney diseases.

In this study, we demonstrated that 4 *coq-2* missense variants conserved between human and *C. elegans* cause varying pathogenic phenotypes consistent with variable severity of primary CoQ10 deficiency in human, and that the phenotypes were generally rescued by CoQ10 supplementation. However, further investigation regarding whether mitochondrial function and morphology are affected by *coq-2* variants remains to be determined. Phenotypic analyses of variants at the subcellular level will help clarify the pathogenicity of undetermined variants and facilitate their interpretation in a clinical context.

DISCLOSURE

All the authors declared no competing interests.

Figure 4. (continued) pigment accumulation may have interfered with rectal muscle function and prevented the rescuing effects of CoQ10 on defecation in *coq-2* variant mutants. All CoQ10 supplementation experiments were performed at a final concentration of 150 μg/ml. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, 2-tailed *t* test. Supplementary Table S7 includes the statistical analysis of the hatching rates, swimming rates, pharyngeal pumping rates, total brood size, developmental time, and defecation cycle data.

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SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Figure S1. Overrepresentation and underrepresentation analysis of 252 ortholog pairs reported with human genetic diseases.

Figure S2. Coenzyme Q2 polyprenyltransferase is a highly conserved protein between human and *C. elegans*.

Figure S3. Predicted pathogenicity and clinical significance of the 4 COQ-2 missense variants of uncertain pathogenicity tested in *C. elegans*.

Table S1. Eight hundred seventy-seven *bona fide* ortholog pairs between human and *C. elegans*.

Table S2. Summary of variants in 252 ortholog pairs associated with human genetic diseases.

Table S3. 101,097 variants in 249 disease-associated human genes reported in the ClinVar.

Table S4. ClinVar variants classified into 5 categories based on clinical significance annotation and types of variants.

Table S5. 44 COQ2 VUS or CIP missense variants in COQ2 that are conserved in *C. elegans*.

Table S6. The clinical information of the 4 COQ-2 missense variants of uncertain pathogenicity tested using *C. elegans* in this study.

Table S7. Statistical analysis of phenotype assays with *C. elegans coq-2* missense variant mutant strains.

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