Genetic studies in *Drosophila* and humans support a model for the concerted function of CISD2, PPT1 and CLN3 in disease

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ABSTRACT

Wolfram syndrome (WFS) is a progressive neurodegenerative disease characterized by diabetes insipidus, diabetes mellitus, optic atrophy, and deafness. WFS1 and WFS2 are caused by recessive mutations in the genes Wolfram Syndrome 1 (WFS1) and CDGSH iron sulfur domain 2 (CISD2), respectively. To explore the function of CISD2, we performed genetic studies in flies with altered expression of its *Drosophila* orthologue, *cisd2*. Surprisingly, flies with strong ubiquitous RNAi-mediated knockdown of *cisd2* had no obvious signs of altered life span, stress resistance, locomotor behavior or several other phenotypes. We subsequently found in a targeted genetic screen, however, that altered function of *cisd2* modified the effects of overexpressing the fly orthologues of two lysosomal storage disease genes, palmitoyl-protein thioesterase 1 (*PPT1* in humans, *Ppt1* in flies) and ceroid-lipofuscinosis, neuronal 3 (*CLN3* in humans, *cln3* in flies), on eye morphology in flies. We also found that *cln3* modified the effects of overexpressing *Ppt1* in the eye and that overexpression of *cln3* interacted with a loss of function mutation in *cisd2* to disrupt locomotor ability in flies. Follow-up multi-species bioinformatic analyses suggested that a gene network centered on CISD2, PPT1 and CLN3 might impact disease through altered carbohydrate metabolism, protein folding and endopeptidase activity. Human genetic studies indicated that copy number variants (duplications and deletions) including *CLN3*, and possibly another gene in the CISD2/PPT1/CLN3 network, are over-represented in individuals with developmental delay. Our studies indicate that *cisd2*, *Ppt1* and *cln3* function in concert in flies, suggesting that CISD2, PPT1 and CLN3 might also function coordinately in humans. Further, our studies raise the possibility that WFS2 and some lysosomal storage disorders might be influenced by common mechanisms and that the underlying genes might have previously unappreciated effects on developmental delay.

**INTRODUCTION**

Wolfram syndrome (WFS) is an autosomal recessive neurodegenerative disease that affects 1 in 770,000 people in the United Kingdom (Barrett et al., 1995). Affected individuals present with diabetes insipidus, diabetes mellitus, optic atrophy and deafness (Wolfram, 1938). Other features of this syndrome include psychiatric illness (Strom et al., 1998) and renal-tract abnormalities (Barrett et al., 1995). Patients usually die within the third decade of life due to respiratory failure associated with brainstem atrophy (Scolding et al., 1996). Mutations in two genes, *WFS1* (Strom et al., 1998) and *CISD2* (Amr et al., 2007) are known to cause WFS1 and WFS2, respectively. *WFS1* encodes wolframin, a transmembrane protein that localizes to the endoplasmic reticulum (ER). Wolframin is important for intracellular calcium homeostasis and is a downstream component of IRE1 and PERK signaling in the unfolded protein response (Osman et al., 2003; Fonseca et al., 2005).

*CISD2*, the second WFS locus, was more recently identified (Amr et al., 2007). A homozygous splice site mutation in *CISD2* that eliminates the full-length transcript was found in three Jordanian families with WFS2 (Amr et al., 2007). *CISD2* encodes a protein with one predicted transmembrane domain and one predicted iron–sulfur domain (Amr et al., 2007; Wiley et al., 2007). Like wolframin, the *CISD2* gene product localizes to the ER (Amr et al., 2007), but whether *CISD2* is involved in regulation of the unfolded protein response has not been addressed. *Cisd2* knockout mice exhibit neurodegeneration along with shortened lifespan (Chen et al., 2009). These mice also have mitochondrial degeneration (Chen et al., 2009), suggesting that *CISD2* is important for mitochondrial integrity and that mitochondrial dysfunction might contribute to the pathology of WFS2. Despite these and other advances in understanding *CISD2*, its function has not been fully resolved. Here, we describe genetic studies in the fruit fly, *Drosophila melanogaster*, and human genetic studies that provide insight into the function of *CISD2*. Our data support a gene network model in which *CISD2* might function in concert with *PPT1*, *CLN3* and several other genes under normal or possibly pathological states.

**RESULTS**

**Identification and RNAi-mediated knockdown of Drosophila cisd2**

BLASTp (Altschul et al., 1997) searches of fly annotated proteins with the predicted gene product of human *CISD2* identified CG1458 as the best orthologue in *Drosophila*. The CG1458 and *CISD2* predicted proteins are 46% identical and 68% similar in
primary amino acid sequence, very similar in size (135 and 133 amino acids, respectively) and have the same predicted topology (Fig. 1A). Additionally, both proteins contain a single predicted transmembrane domain (Fig. 1A) as well as a single predicted CDGSH iron–sulfur domain at the same position (Fig. 1B). BLASTp (Altschul et al., 1997) searches of human annotated proteins with the fly CG1458 translation product identified CISD2 as the best human orthologue, although the human CISD1 locus encodes a protein that also shares considerable homology to that of CG1458 (31% identical, 48% similar, single predicted transmembrane domain, single CDGSH iron–sulfur domain). Considering these data, and that we found no other predicted fly proteins with significant homology to the CISD2 gene product, we have designated CG1458 in Drosophila as cisd2.

We used the Gal4-UAS system (Brand and Perrimon, 1993) to drive two RNA interference (RNAi) transgenes to manipulate cisd2 expression. da-Gal4-driven ubiquitous expression of UAS-cisd2-RNAi transgenes v33925 and v33926 (Dietzl et al., 2007) decreased cisd2 mRNA levels by 99±0.1% and 97±0.4%, respectively (n=3) as determined by quantitative real-time PCR (qRT-PCR). The cisd2 RNAi transgenes do not have predicted off-target effects (see Materials and Methods) and do not alter expression of Drosophila wfs1 (the fly orthologue of the causative gene for WFS1, data not shown). A protein band consistent with the size of the cisd2 translation product was readily detectable on immunoblots of extracts from control flies, but not from flies with ubiquitous expression of cisd2 RNAi transgenes v33925 or v33926 (Fig. 2A). These qRT-PCR and immunoblot results indicate that expression of the cisd2 RNAi transgenes causes a strong loss of function in cisd2, although they do not rule out the possibility that some residual expression of cisd2 remains in these animals.

**Knockdown of cisd2 alone does not have obvious detrimental effects in Drosophila**

As an initial step toward a genetic analysis of cisd2 in Drosophila, we determined whether knocking down its

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**Fig. 1. Conserved structure of the CISD2 and cisd2 gene products.** (A) Hydropathy plots for gene products from human CISD2 (i) and fly cisd2 (ii). Amino termini are on the left. Predicted transmembrane domains (TMpred at embnet) are indicated by arrows. (B) Comparison of the primary amino acid sequences for the two gene products. Gaps are represented by asterisks (*). Identical amino acids are in bold. The single underline represents the predicted transmembrane domains. The double underline represents the CDGSH domains.
expression in several different tissues led to obvious phenotypes in adults reared and aged under normal housing conditions. Knockdown of cisd2 throughout the body (da-Gal4, Fig. 2B,C; Actin-Gal4, supplementary material Table S2), in the musculature (me2-Gal4, supplementary material Table S2), or in the nervous system (elav-Gal4, 188Y-Gal4 and Appl-Gal4, supplementary material Table S2) had no obvious effects on lifespan or age-related locomotor impairment. Additionally, knockdown of cisd2 ubiquitously (da-Gal4), in the musculature (me2-Gal4) or in the nervous system (Appl-Gal4) did not lead to a change in bang sensitivity (an index of seizure susceptibility (Fergestad et al., 2006)) in 1–8-week-old flies (supplementary material Table S2). Ubiquitous knockdown of cisd2 via da-Gal4 had no significant effect on expression of 4E-BP at 1 or 8 weeks of age (supplementary material Table S2), suggesting that insulin signaling was not impaired in these animals (Fuss et al., 2006). Expression of cisd2 RNAi in the eye via gmr-Gal4 (Freeman, 1996) also had no discernible effect on external eye morphology (Fig. 3A–C). Thus, knockdown of cisd2 via a number of Gal4 drivers does not appear to have major negative consequences on several independent measures in flies housed under normal laboratory conditions.

To address whether cisd2 might be important for stress sensitivity in Drosophila, we evaluated whether ubiquitous knockdown of cisd2 altered survival when flies were exposed to thermal, desiccation, starvation, oxidative (hyperoxia, paraquat and H2O2), FeCl3 (iron overload) and tunicamycin (ER) stress. We assessed survival of flies at 1 and 6 weeks of adulthood to address the possibility that effects of cisd2 knockdown might manifest with age. Although we occasionally saw subtle effects of ubiquitous knockdown of cisd2 on stress sensitivity in individual experiments, these effects were not consistently observed (supplementary material Table S2). Additionally, expression of cisd2 RNAi selectively in the nervous system and musculature had no consistent effect on sensitivity to exogenous stressors (supplementary material Table S2). Knockdown of cisd2, therefore, had no discernible effect on sensitivity to any of the stressors we tested.

**Targeted genetic analysis identifies a novel interaction between cisd2 and Ppt1**

Given that mutations in CISD2 cause neurodegeneration in WFS2, we postulated that cisd2 might interact with genes known or predicted to cause other forms of neuropathology in flies. We therefore assessed whether gmr-Gal4-driven expression of cisd2 RNAi modified the phenotypes in several genetic models of neurodegeneration (autosomal dominant retinitis pigmentosa, ataxia telangiectasia, Parkinson disease, Alzheimer disease, etc., supplementary material Table S3). Additionally, we determined whether gmr-Gal4-driven expression of cisd2 RNAi led to a
synthetic phenotype in conjunction with altered cellular processes associated with pathology (oxidative stress, apoptosis and autophagy, supplementary material Table S3).

Light microscopic analyses in a small-scale screen with ~50 flies/genotype suggested that knockdown of cisd2 modified the external eye morphology in two strains that overexpressed wild-type Drosophila palmitoyl-protein thioesterase 1 (Ppt1). Knockdown of cisd2, however, had no discernible effect in any of the other strains tested (supplementary material Table S3). Overexpression of Ppt1 in the Drosophila eye causes blackened ommatidia thought to be indicative of apoptosis (Korey and MacDonald, 2003). In humans, loss of function mutations in PPT1 cause infantile neuronal ceroid lipofuscinosis, a severe pediatric neurodegenerative disease resulting in death by 10 years of age (Vesa et al., 1995).

We pursued the possibility that knockdown of cisd2 modifies the Ppt1 overexpression phenotype by performing a series of larger single-blind studies that included more than 300 eyes per genotype. As previously reported (Korey and MacDonald, 2003), we found that overexpression of Ppt1 in the fly eye via two independent transgenes (Ppt1-2.1 and Ppt1-8.1) led to a blackened ommatidia phenotype with variable expressivity (Fig. 3D–G). We formally quantitated the severity of the black ommatidia phenotype in each eye using a four-point scale: normal (no black ommatidia, 0); slight (a few black ommatidia, 1); mild (one or more small patches of black ommatidia, 2); or moderate (black ommatidia throughout the eye, 3) (Fig. 3D–G). We then compiled the data for genotypes expressing Ppt1 alone or concurrently with the cisd2 RNAi transgenes. These larger studies confirmed that cisd2 knockdown (via the v33925 RNAi transgene) partially suppressed (i.e. decreased the severity of) the black ommatidia phenotype due to overexpression of two independent Ppt1 transgenes (Fig. 4A, Ppt1-2.1; Fig. 4C, Ppt1-8.1). Similarly, knockdown of cisd2 with the v33926 RNAi transgene led to a partial suppression of the black ommatidia phenotype in flies overexpressing Ppt1 (Fig. 4B, Ppt1-2.1; Fig. 4D, Ppt1-8.1).

To address the possibility that expression of the cisd2 RNAi transgenes suppressed the severity of black ommatidia simply by blunting the overexpression or function of Ppt1, we evaluated Ppt1 mRNA expression and enzyme activity. We used fly head extracts for these studies because gmr-Gal4 drives expression in the eye (a major portion of the head). Expression of cisd2 RNAi had no discernible effect on Ppt1 mRNA overexpression (Fig. 5A). Additionally, although expression of the v33925 RNAi transgene led to a modest but statistically discernible decrease in PPT1 enzyme activity (Fig. 5B), expression of the v33926 RNAi transgene did not alter PPT1 enzyme activity (Fig. 5C). The most parsimonious interpretation of our mRNA and enzyme activity studies is that the cisd2 RNAi-mediated suppression of black ommatidia is unlikely to be due to decreased expression or function of Ppt1.

We used a transposon insertion mutation (P{EP}G6528) that resides within the protein coding sequence of cisd2 exon 1 (http://flybase.org) to further address the possibility that cisd2 influences the effects of Ppt1 overexpression in the eye. We confirmed the reported location of the G6528 insertion in exon 1 of cisd2 using standard PCR on genomic DNA and also found that G6528 reduced cisd2 expression to nearly undetectable levels (2.6% of control, one sample t test, p<0.0001, n=3). The insertion site and decreased cisd2 mRNA expression indicate that G6528 is a very strong loss of function allele of cisd2. Consistent with our RNAi data (Fig. 4), the black ommatidia phenotype from Ppt1 overexpression was greatly reduced in a cisd2G6528 background (Fig. 3H,I, Fig. 6A). These data confirm that loss of function in cisd2 modifies the Ppt1 overexpression eye phenotype.

cisd2 exhibits a genetic interaction with cln3

Given that cisd2 is a genetic modifier of Ppt1, the fly orthologue of human PPT1, we postulated that cisd2 may interact with additional genes associated with lysosomal storage diseases. We therefore assessed the effect of cisd2 knockdown on the external eye morphology of flies with altered expression of or mutations in genes associated with several different lysosomal storage diseases (supplementary material Table S4). In initial experiments using light microscopy, knockdown of cisd2 appeared to enhance the disorganized ommatidia phenotype caused by overexpression of

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**Fig. 4.** cisd2 RNAi suppresses the effects of Ppt1 overexpression in the eye. gmr-Gal4-driven expression of two independent UAS-Ppt1 transgenes (Ppt1-2.1 and Ppt1-8.1) alone or with cisd2 RNAi caused blackened ommatidia that varied in severity (representative photographs in Fig. 2). The severity of black ommatidia in each eye was scored on a four-point scale for quantification: none (0), slight (1), mild (2) and moderate (3). (A,B) gmr-Gal4/UAS-PPT1-2.1 alone, with v33925 or with v33926. (C,D) gmr-Gal4/UAS-PPT1-8.1 alone, with v33925 or with v33926. Expression of v33925 or v33926 cisd2 RNAi decreased the severity of Ppt1-induced black ommatidia in all cases (individual Mann–Whitney tests, p<0.0001, n=310–396 per genotype). Data are compiled from two independent experiments.
Drosophila cln3 (data not shown), the orthologue of human ceroid-lipofuscinosis, neuronal 3 (CLN3) (Tuxworth et al., 2009). In humans, mutations in CLN3 cause a juvenile form of neuronal ceroid lipofuscinosis, a lysosomal storage disease. Consistent with our initial RNAi data, the disorganized ommatidia and loss of eye bristles seen with cln3 overexpression were exacerbated in a cis2ΔG6528 mutant background as determined by light (Fig. 3H,J,K) and scanning electron microscopy (Fig. 3L–N).

**cis2** loss of function and **cln3** gain of function cause a synthetic locomotor phenotype

We addressed the possibility that cis2, Ppt1 and cln3 might interact in tissues outside of the eye by assessing negative geotaxis in flies overexpressing Ppt1 or cln3 throughout the nervous system in cis2 wild-type or mutant backgrounds (Fig. 6B). Negative geotaxis was not altered in cis2ΔG6528 mutants compared to our standard w1118 laboratory stock, consistent with our previous cis2 RNAi studies (Fig. 2; supplementary material Table S2). elav-Gal4-driven nervous system overexpression of Ppt1 in a cis2 wild-type or mutant background did not affect negative geotaxis, precluding a formal assessment of a possible genetic interaction between these two genes within the context of this behavior. Interestingly, while negative geotaxis was normal in cis2ΔG6528 mutants and in flies overexpressing cln3, flies with concurrent cis2 loss of function and cln3 gain of function had substantial decreases in this behavior (Fig. 6B). This cis2/cln3 synthetic phenotype is consistent with our studies showing that cis2 loss of function enhances the effect of cln3 overexpression in the eye (Fig. 3H,J–N).

**Knockdown of cis2 does not interact with loss of function in Ppt1, cln3 or related genetic modifiers**

Since reduced function of Ppt1 or cln3 alone does not cause obvious changes in external eye morphology (Hickey et al., 2006; Tuxworth et al., 2009), we postulated that cis2 knockdown in conjunction with Ppt1 or cln3 loss of function might lead to a synthetic phenotype in this tissue. Similarly, we postulated that cis2 may work in concert with previously identified genes that genetically interact with Ppt1 and cln3 (Buff et al., 2007; Tuxworth et al., 2009). We therefore evaluated the external eye morphology in flies harboring loss of function in Ppt1, cln3 or previously reported genetic modifiers of these genes (supplementary material Table S5) alone and with cis2 knockdown. Knockdown of cis2 in the eye did not lead to obvious changes in the external morphology of the eye in any of these additional studies. These studies suggest that reduced function of cis2 does not interact with Ppt1 or cln3 loss of function manipulations or genetic modifiers of Ppt1 or cln3. Our studies do not formally rule out these possibilities, however, since our interpretation is based on the lack of a synthetic phenotype.

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Fig. 5. Effect of RNAi-mediated knockdown of cis2 on Ppt1 mRNA expression and enzyme activity. (A) Expression of v33925 did not alter total head Ppt1 mRNA expression (individual t tests, n.s., n=4). (B,C) PPT1 activity in fly heads. (B) Co-expression of the v33925 RNAi transgene with Ppt1-2.1 or Ppt1-8.1 decreased PPT1 enzyme activity (*individual t tests, n=9 per genotype, p=0.019 and p=0.002, respectively). (C) Co-expression of the v33926 RNAi transgene with either Ppt1-2.1 or Ppt1-8.1 did not affect PPT1 enzyme activity (individual t tests, n=17–18, n.s.). All data are presented as fold increases relative to endogenous Ppt1 mRNA or PPT1 activity in heads from gmr-Gal4+ control flies.

Fig. 6. A cis2 loss of function mutation interacts with Ppt1 and cln3 overexpression. (A) The severity of black ommatidia in flies expressing Ppt1 was reduced by the cis2ΔG6528 mutation (*Mann–Whitney test, p<0.0001, n=208–237). (B) Genotype had a significant overall effect on negative geotaxis (one-way ANOVA, p<0.0001, n=6–9). The cis2ΔG6528 mutation decreased negative geotaxis in flies overexpressing cln3 in the nervous system (*Bonferroni multiple comparison, p<0.001).
A novel interaction between Ppt1 and cln3

Our studies in flies indicate that Ppt1 and cln3 exhibit genetic interactions with cis2, suggesting that Ppt1 and cln3 may be functionally linked. To address this possibility, we evaluated external eye morphology in flies expressing several different combinations of Ppt1 and cln3 transgenes. We found that cln3 RNAi (v5322) partially enhanced while cln3 overexpression (venus-cln3 or cln3) partially suppressed the severity of black ommatidia caused by Ppt1 overexpression (Fig. 7A). Conversely, we found no evidence that Ppt1 overexpression or loss of function altered the rough eye phenotype due to cln3 overexpression (not shown). Eyes with concurrent overexpression of both Ppt1 and cln3 did exhibit a decrease in pigmentation, but this change could be due to simple additive pathology. These data indicate that cln3 modifies the Ppt1 black ommatidia phenotype in Drosophila and raise the possibility that cln3 might normally function as a negative regulator of the Ppt1 pathway in flies.

Genetic and gene network models for the coordinated function of cis2, Ppt1 and cln3

Our data support a model in which cis2, Ppt1 and cln3 function in concert in flies (Fig. 7B). In this model, endogenous cis2 is a positive regulator of the pathway leading from Ppt1 overexpression to black ommatidia while it is a negative regulator of the pathway leading from cln3 overexpression to disorganized ommatidia. Additionally, cln3 antagonizes the Ppt1 pathway leading to black ommatidia in our model. Importantly, the arrows in our model (Fig. 7B) could represent any number of genes involved in the eye phenotypes caused by overexpression of Ppt1 or cln3. Our studies are the first to support a model for the coordinated function of cis2, Ppt1 and cln3 in any species.

To better understand the model in Fig. 7B, we used gene network analyses in GeneMania (Mostafavi et al., 2008; Warde-Farley et al., 2010) to identify known or predicted pair-wise gene interactions for orthologues of cis2, Ppt1 and cln3 (i.e. seed genes) in S. cerevisiae, C. elegans, Drosophila, mice and humans. Interactions in GeneMania are defined by gene pairs that are co-expressed, have demonstrated or predicted genetic interactions, or encode gene products that physically interact, have shared protein domains or co-localize (Mostafavi et al., 2008; Warde-Farley et al., 2010).

We compiled all genes from GeneMania known or predicted to interact with orthologues of cis2, Ppt1 and cln3 in humans, mice, flies, worms and yeast (supplementary material Table S6) and then converted all of the interacting orthologues to human gene symbols for convenience (supplementary material Table S7) (gProfiler; Reimand et al., 2011). The resulting multi-species interaction gene network contains 117 human genes total, with 99 genes known or predicted to interact with CIDS2, PPT1 or CLN3 (the human orthologues of fly cis2, Ppt1 and cln3, respectively) (Fig. 8A). Approximately one-third of the gene–gene interactions in the CIDS2/PPT1/CLN3 multi-species interaction network were based on co-expression data, while the remainder of the interactions was based on other results (supplementary material Table S8). We identified 32 genes (collectively from the five species queried) that interacted with two seed genes used to derive the network (supplementary material Table S9). The CIDS2/PPT1/CLN3 multi-species interaction network as a whole is over-represented for genes involved in carbohydrate metabolism, chaperone/protein folding and endopeptidases/peptidases (Huang et al., 2009a; Huang et al., 2009b), suggesting that these processes might underlie disease states associated with altered function of CIDS2, PPT1 and CLN3.

To address the possibility that the CIDS2/PPT1/CLN3 network – and more specifically the gene–gene interactions that defined it – arose from random chance, we compared gene–gene interactions between CIDS2/PPT1/CLN3 network genes derived from human data (supplementary material Table S6) to interactions within 120 additional GeneMania networks seeded with randomly selected sets of human genes (supplementary material Table S10). The randomly selected seed genes (in sets of 3 to mirror the scope of the seed genes in the CIDS2/PPT1/CLN3 network) were from (i) the genes or orthologues of genes in supplementary material Table S3 that showed no interaction with knockdown of fly cis2 and (ii) annotated genes from the human genome as a whole (Meyer et al., 2013). The total number of genes was somewhat higher (Fig. 8B) while the number of genes that interacted with seed genes was comparable (Fig. 8C) in the randomly generated and the CIDS2/PPT1/CLN3 networks. Strikingly, the number of genes that interacted with two of the three seed genes (i.e. bivalent interactors) was substantially higher in the CIDS2/PPT1/CLN3 network compared to the 120 networks seeded with randomly selected genes (Fig. 8D). This analysis indicates that the CIDS2/PPT1/CLN3 network has a...
more complex, multi-valent structure around the seed genes than would be expected by chance alone. Therefore, this network could be informative regarding the collective function of \textit{CISD2}, \textit{PPT1} and \textit{CLN3}.

Given the severe clinical neuropathology in patients with mutations in \textit{CISD2}, \textit{PPT1} and \textit{CLN3} and the genetic interactions between these genes in flies, we postulated that the \textit{CISD2/PPT1/CLN3} gene network might be broadly involved in human neurological conditions. To examine this gene network specifically in neurodevelopmental disorders, we searched human genetic data for variants within \textit{CISD2}, \textit{PPT1}, \textit{CLN3} and the other 19 human genes identified by network analysis in humans (supplementary material Table S6). Individually rare but collectively common CNVs are known to be enriched in cases with neurodevelopmental disorders including intellectual disability and congenital malformations, autism, schizophrenia, congenital cardiac disease and epilepsy (Girirajan and Eichler, 2010; Girirajan et al., 2011). We compared the frequencies of rare CNVs (deletions and duplications combined) encompassing \textit{CISD2}, \textit{PPT1}, \textit{CLN3} and their gene network partners in unaffected controls and in individuals with intellectual disability phenotypes. Depending on the probe coverage sufficient to make high confidence CNV calls, the total number of cases evaluated ranged from 8,300 to 58,120 (Table 1). We found a small but significant overall enrichment for rare variants for other genes in the cases compared to controls.
Seed genes were used to generate a gene network in GeneMania that contained the interacting genes listed.

**DISCUSSION**

WSF1 and WSF2 are caused by mutations in *WSF1* (Ström et al., 1998) and *CISD2* (Amr et al., 2007), respectively. Although we are beginning to better understand the biochemical properties of the *CISD2* gene product (Amr et al., 2007), the genes and genetic pathways associated with *CISD2* have not been characterized. Here, we performed a series of genetic and bioinformatic analyses to identify molecular pathways associated with *CISD2* function.

We used RNAi and a mutation to determine whether decreased function of cisd2 (the fly orthologue of *CISD2*) led to obvious phenotypes in *Drosophila*. Surprisingly, flies with strong loss of function in cisd2 alone appeared remarkably healthy under standard housing conditions and when subjected to various exogenous stressors. While we do not currently understand why flies with cisd2 knockdown were seemingly unperturbed, several possibilities exist. One possibility is that cisd2 knockdown could be deleterious only under prescribed environmental conditions such as in the presence of certain microbial pathogens as found in *Drosophila*. Surprisingly, flies with strong loss of function entirely retain wild-type eye phenotypes in several previously described models of mutant phenotypes in *Drosophila*. At this time, we can only speculate about the mechanistic connections between *CISD2*, *PPT1*, *CLN3* and their network genes is another possibility is that *PPT1*-mediated de-palmitoylation of *CLN3* and a 1.02 kb deletion on the non-deleted allele of *CLN3*. This individual showed features of juvenile ceroid lipofuscinosis or Batten disease in addition to features of developmental delay, attention deficit disorder, and seizures (Pebrel-Richard et al., 2014). Notably, *CLN3* is frequently deleted or duplicated in individuals carrying atypical CNVs involving either the autism or the obesity-associated regions. Recently, Pebrel-Richard and colleagues reported a case with a large heterozygous deletion on chromosome 16p11.2 encompassing *CLN3* and a 1.02 kb deletion on the non-deleted allele of *CLN3*. This individual showed features of juvenile ceroid lipofuscinosis or Batten disease in addition to features of developmental delay, attention deficit disorder, and seizures (Pebrel-Richard et al., 2014). Together, these studies suggest that disruption of *CISD2* function in concert. Additionally, our gene network analyses suggest that cisd2, Ppt1 and cln3 (and their orthologues in *S. cerevisiae*, *C. elegans*, mice and humans) might be functionally connected to many other genes, including genes that regulate carbohydrate metabolism, chaperone/protein folding and endopeptidases/proteases.

Our studies found that CNVs encompassing human *CLN3* and *SCAMP2* are associated with neurodevelopmental disorders. *CLN3* maps to chromosome 16p11.2 distal to regions previously associated with developmental delay, autism and obesity (Weiss et al., 2008; Bachmann-Gagescu et al., 2010; Bochukova et al., 2010; Rosenfeld et al., 2010; Walters et al., 2010). Notably, *CLN3* is frequently deleted or duplicated in individuals carrying atypical CNVs involving either the autism or the obesity-associated regions. Recently, Pebrel-Richard and colleagues reported a case with a large heterozygous deletion on chromosome 16p11.2 encompassing *CLN3* and a 1.02 kb deletion on the non-deleted allele of *CLN3*. This individual showed features of juvenile ceroid lipofuscinosis or Batten disease in addition to features of developmental delay, attention deficit disorder, and seizures (Pebrel-Richard et al., 2014). Together, these studies suggest that disruption of *CLN3* or possibly other genes in the *CISD2/PPT1/CLN3* interaction network could play a role in several pathological states.

At this time, we can only speculate about the mechanistic connections between *CISD2*, *PPT1*, *CLN3* and their network genes. One possibility is that PPT1-mediated de-palmitoylation of the gene products for *CISD2*, *CLN3* and other network genes is important for their degradation or subcellular localization and therefore function (Smetros and Linder, 2004). Another possibility is that *CLN3*-mediated signaling via Notch and JNK or synthesis of sphingolipids (Buff et al., 2007; Persaud-Sawin et al., 2007; Tuxworth et al., 2009) might be important for the function of *CISD2*, *PPT1* or other genes in the network. Yet another possibility is that *CISD2*, *PPT1* and *CLN3* are functionally connected via one or more of the other genes in the network through an as yet unidentified biochemical pathway. Our studies provide the rational framework for further

| Class | Chrom | Start | End | Gene | Case CNVs | Denominator | Control CNVs | p-value (one-tailed) | OR |
|-------|-------|-------|-----|------|-----------|-------------|--------------|---------------------|----|
| Seed genes | chr4 | 104009576 | 104033412 | CISD2 | 3 | 33504 | 2 | 0.9428 | 0.2485 |
| chr1 | 40310969 | 40335729 | PPT1 | 7 | 33504 | 0 | 2.114 | 1.7401 |
| chr16 | 28396101 | 28411124 | CLN3 | 42 | 42711 | 0 | 0.0006* | 8.1964 |
| Interacting genes | chr1 | 20850847 | 20860624 | DDOST | 3 | 33504 | 1 | 0.8206 | 0.3728 |
| chr1 | 40496309 | 40532443 | ZMPS2E24 | 7 | 33504 | 0 | 2.114 | 1.7401 |
| chr1 | 45869042 | 46088310 | AKR1A1 | 5 | 33504 | 0 | 0.2595 | 1.2429 |
| chr1 | 143807764 | 144382354 | SEC22B | 2 | 8300 | 0 | 0.1805 | 1.7007 |
| chr1 | 177529472 | 177954437 | SOAT1 | 12 | 33504 | 8 | 0.9903 | 0.3312 |
| chr10 | 59698888 | 59719025 | CISD1 | 7 | 33504 | 0 | 0.2114 | 1.7401 |
| chr11 | 71605467 | 71610642 | POLR2 | 0 | n/a | n/a | n/a | n/a |
| chr14 | 23804584 | 23810673 | RABGTA | 7 | 42711 | 0 | 0.2874 | 1.3650 |
| chr14 | 61231872 | 61284730 | HIF1A | 3 | 42711 | 3 | 0.9920 | 0.1462 |
| chr15 | 72924250 | 72952723 | SCAMP2 | 29 | 42711 | 1 | 0.0327* | 2.8285 |
| chr16 | 30951917 | 30958889 | STX4 | 9 | 42711 | 1 | 0.4968 | 0.8774 |
| chr16 | 31027116 | 31036131 | BCKDK | 8 | 42711 | 1 | 0.5544 | 0.7799 |
| chr17 | 34140036 | 34143843 | CISD3 | 3 | 42711 | 0 | 0.5860 | 0.5849 |
| chr17 | 37864388 | 37928123 | ATP6V0A1 | 2 | 42711 | 1 | 0.9288 | 0.1949 |
| chr17 | 39778017 | 39785996 | GRN | 2 | 33504 | 5 | 0.9996 | 0.0828 |
| chr19 | 150865785 | 15097577 | ILVBL | 6 | 42711 | 0 | 0.3434 | 1.1699 |
| chr2 | 32229754 | 32239436 | PPT2 | 2 | 42711 | 0 | 0.7003 | 0.3899 |
| chr2 | 66009025 | 66098023 | SBDS | 5 | 33504 | 0 | 0.2395 | 1.2429 |
| chr8 | 145509055 | 145521375 | DGAT1 | 40 | 58120 | 3 | 0.1957 | 1.432 |

Seed genes were used to generate a gene network in GeneMania that contained the interacting genes listed.
investigating these possibilities and therefore the functional connections between genes in the CISD2/PPT1/CLN3 network. Such studies could lead to a better understanding of the pathogenesis of WFS, lysosomal storage diseases and neurodevelopmental disorders.

**MATERIALS AND METHODS**

**Drosophila husbandry, strains, and genetics**

Fly husbandry and aging were performed as described (Gargano et al., 2005). da-Gal4, me2-Gal4, 1887-Gal4, app-Gal4, cis2G6528 and all Ppt1 modifiers listed in supplementary material Table S5 were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN). UAS-RNAi transgenic lines for cis2 (v33925 and v33926), cln3 (v3522) and cln7 (v5089 and v5090) were purchased from the Vienna Drosophila RNAi Center (Vienna, Austria) (Dietzl et al., 2007). The cis2 RNAi transgenes do not have predicted off-target effects (defined as genes with at least one continuous stretch of 19 nucleotides complementary to any transgenes do not have predicted off-target effects (defined as genes with at least one continuous stretch of 19 nucleotides complementary to any region of the RNAi transgene (http://stockcenter.vdrc.at/control/vrdedefinition)). The gmr-Gal4-v33925 and the gmr-Gal4-v33926 double transgenic flies were created by recombining the gmr-Gal4 element and the cis2 RNAi transgenes onto the same chromosome. UAS-Ppt1 transgenic strains are previously described (Korey and MacDonald, 2003). Ppt1 loss of function mutant strains (Ppt1/179T and Ppt1/77F) were provided by Robert Glaser (Wadsworth Center, Albany NY). The cln3 overexpression strain (UAS-cln3 no. 4) was provided by Richard Tuxworth (Kings College London, London, UK). Sources for all other strains are indicated in supplementary material Tables S3, S4, S5.

**Drosophila behavioral assays**

Negative geotaxis (startle-induced climbing) was analyzed in Rapid Iterative Negative Geotaxis (RING) assays as described previously (Gargano et al., 2005) with 125 animals per genotype. Lifespan was assessed as previously described (Martin et al., 2009). Bang sensitivity was assessed by determining the climbing latency (i.e. time to recovery) in groups of 25 flies after being vortexed in a vial for 15 seconds at the highest setting using a Diagger Vortex Genie 2 (Fergestad et al., 2006).

**Quantitative real-time PCR**

mRNA expression was assessed via quantitative real-time PCR (qRT-PCR) studies as previously described (Jones et al., 2009). Briefly, groups of 25 male flies or ∼800 fly heads were frozen at −80°C. Total RNA was isolated using TRIZOL (Invitrogen) and reverse transcribed using oligo(dT) primers and Superscript II reverse transcriptase (Invitrogen). qRT-PCR was performed using an Applied Biosystems Fast 7500 system with SYBR Green PCR master mix (Quanta Biotics). All SYBR green assays were performed in triplicate and normalized to Actin5c mRNA expression. Each qRT-PCR experiment was repeated three times with three independent RNA isolations and cDNA syntheses. Primer information is listed in supplementary material Table S1.

**Drosophila stress tests**

All flies for stress tests were collected at 1–3 days of age and were tested for stress sensitivity at 1 and 6 weeks of age. In each experiment, the number of dead flies was recorded for each stress test every 4–8 hrs until all flies were dead. Three vials of 25 flies each were tested for each group. For starvation studies, flies were housed in food vials containing 1% agar. For desiccation studies, flies were housed in empty vials placed in a box with desiccant. To assess thermal stress, flies were placed in vials containing 1% agar with 5% sucrose at 36°C incubator. Hyperoxia studies were performed by placing flies in standard food vials in an air tight container charged with 95% O2 twice daily. All drug tests compared survival in drug-treated and vehicle-treated food vials. For Tunicamycin treatment, flies were placed into vials with food pre-treated with 100 μl 2 mM Tunicamycin in 95% ethanol or 95% ethanol (vehicle). Flies were exposed to paraquat, FeCl3, and H2O2 by placing them in vials with 2 Whatman paper discs treated with 300 μl of 5% sucrose (vehicle) or 5% sucrose supplemented with 40 mM paraquat, 200 mM FeCl3, or 30% H2O2, respectively.

**PPT1 activity assay**

PPT1 enzyme activity levels were measured as described previously (Buff et al., 2007). Briefly, a single fly head was placed in a well of a 96 well plate on ice with ~15 heads used per genotype. Heads were crushed by a pestle in 30 μl solution consisting of 20 μl H2O and 10 μl of the PPT1 fluorogenic substrate (4-MU-6S-palm-β-glc) and incubated for 2 hours at 30°C. PPT1 activity was measured by the absorbance change at 460 nm. Ppt1 loss of function flies were used as a negative control.

**Immunobots**

Protein was isolated from 25 flies per genotype by homogenization in radioimmunoprecipitation RIPA lysis buffer containing protease inhibitor cocktail (Roche 1:25 dilution in lysis buffer). Samples were sonicated, incubated on ice for 45 minutes and centrifuged at 16,000 × g for 15 minutes at 4°C. Supernatants were transferred to a new tube and protein concentration was measured using the DC Protein Assay (Bio-Rad). Protein extracts were electrophoresed via SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Western blots were probed with a rabbit anti-mouse polyclonal antibody against the CISD2 gene product (ProteinTech, 1:1,000 dilution in 5% BSA in Tris-buffered saline solution containing 0.1% Tween-20 (TBST) to detect Drosophila WFS2 (dWFS2) or a mouse anti-α tubulin monoclonal antibody (Sigma, 1:1,000 dilution in 5% milk in TBST) to detect the loading control. Expression of dWFS2 and α-tubulin was visualized with goat anti-rabbit IgG-HRP (BioRad, 1:10,000 dilution in 5% milk in TBST) and goat anti-mouse IgG-HRP (Santa Cruz, 1:10,000 dilution in 5% milk in TBST), respectively, in conjunction with Western Lightning chemiluminescence reagent plus (PerkinElmer). Western blot experiments were repeated three times with independent protein extracts.

**Light and electron microscopy**

Samples for and images of external eye morphology were processed as previously described (Warrick et al., 1999; Chan et al., 2002).

**Gene network analyses**

Gene networks were constructed using GeneMania (Mostafavi et al., 2008; Warde-Farley et al., 2010) with default settings. Interactors from GeneMania, including genes that interacted with more than one seed gene, were identified using Excel (Microsoft, Redmond, WA). Identification of orthologues in S. cerevisiae, C. elegans, Drosophila, mice and humans was performed using g:Profiler (Reimand et al., 2011) and BLASTp (Altschul et al., 1997). Random sets of 3 genes were selected from supplementary material Table S3 and the human genome by sorting the relevant gene list based on a randomly assigned number in Excel. Gene ontology analysis was performed with DAVID (Huang et al., 2009a; Huang et al., 2009b).

**Human disease-associated variation**

To examine CLN3, PPT1, CISD2 and a set of 19 of their interacting partners in the context of a broader neurodevelopmental phenotype, we evaluated human disease-associated variation data from exome sequencing and copy number variation analysis. Specifically, disruptive de novo single nucleotide mutations within the 22 genes were queried in the exome sequencing data from 151 families with severe intellectual disability (de Ligt et al., 2012; Rauch et al., 2012) and 927 families with sporadic autism (O’Roak et al., 2011; Iossifov et al., 2012; Neale et al., 2012; O’Roak et al., 2012; Sanders et al., 2012). We also analyzed CNV data from a clinical laboratory database consisting of 58,120 individuals referred primarily for intellectual disability, developmental delay, and other congenital malformations for deletions and duplications within the genes of interest. These samples from affected individuals were sent to Signature Genomic Laboratories from 2004 through 2013 by geneticists, pediatricians, and neurologists from more than 50 referral centers primarily throughout the United States. The ages of the ascertained cases ranged between 2 to 22 years. Based on self-reported ethnicity, about
75% are of European descent, 7% African or African-American, and 18% belonged to other or mixed ancestry (Cooper et al., 2011). These samples were evaluated by array comparative genomic hybridization (array CGH) experiments with a targeted whole genome bacterial-artificial-chromosomal microarray (SignatureChip) or an oligonucleotide-based microarray (SignatureChipOS, custom-designed by Signature Genomic Laboratories and manufactured by Agilent Technologies or Roche NimbleGen). Microarray hybridizations were performed as described previously (Bejiani et al., 2005; Ballif et al., 2008a; Ballif et al., 2008b; Duker et al., 2010). Control CNV data were curated from single nucleotide polymorphism arrays from 8329 individuals with no overt neurological disorders as described previously (Cooper et al., 2011).

We only included those CNVs in the affected individuals that are rare (<0.1% frequency in controls), large (>300 kb), <50% overlapped with large genomic repeats called segmental duplications, and mapped to putative genes described in this study. Further, we only considered interstitial heterozygous deletions and duplications. Large chromosomal abnormalities such as trisomies and monosomies were excluded from the analysis. We considered all CNVs that overlapped by at least 1 bp with the putative gene of interest and compared frequency of events hitting the genes of interest between cases and controls. Depending on the probe coverage of the genes evaluated, the total number of cases available for analysis ranged from 8,300 to 58,120 individuals (Table 1).

Experimental subjects

The CNV data were curated from a database in Signature Genomic Laboratories. CNVs from de-identified samples were analyzed for variants in specific genes of interest. All experiments with human data conform to the relevant regulatory standards.

Statistics

JMP 5.0.1a (SAS Institute, Cary, NC) was used to analyze lifespan and stress survival data (log-rank tests) and negative geotaxis across age (two-way ANOVA). The severity of black ommatidia (categorical data) was analyzed with nonparametric Mann-Whitney tests or Kruskal-Wallis ANOVA followed by Dunn’s multiple comparison using Prism 4.03 (GraphPad Software, San Diego, CA). Data for Ppt1 mRNA, PPT1 enzyme activity and negative geotaxis at a single age were analyzed with parametric t tests. Statistical analyses on human data were performed with the hypothesis that rare CNVs encompassing genes of interest would be enriched in cases compared to controls and thus one-tailed Fisher’s exact tests were used.

Resource sharing

Enquiries for reagents described in this article should be directed to the corresponding author (M.G.).

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Competing interests

J.A.R. is an employee of Signature Genomics (Spokane, WA, USA).

Author contributions

M.A.J., S.A., M.F.M., A.G.D., C.A.K., J.M.W., R.S., S.H.E., S.G. and M.G. developed the concepts and approaches. M.A.J., S.A., A.F., P.H., J.A.R., A.G.D., C.A.K., J.M.W., R.S. and M.G. performed the experiments and analyzed the data. M.A.J., J.A.R., M.W. and S.G. wrote the manuscript.

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