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The Batten disease gene *CLN3* is required for the response to oxidative stress

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Mutations in the *CLN3* gene cause juvenile neuronal ceroid lipofuscinosis (JNCL or Batten disease), an early onset neurodegenerative disorder. JNCL is the most common of the NCLs, a group of disorders with infant or childhood onset that are caused by single gene mutations. The NCLs, although relatively rare, share many pathological and clinical similarities with the more common late-onset neurodegenerative disorders, while their simple genetic basis makes them an excellent paradigm. The early onset and rapid disease progression in the NCLs suggests that one or more key cellular processes are severely compromised. To identify the functional pathways compromised in JNCL, we have performed a gain-of-function modifier screen in *Drosophila*. We find that *CLN3* interacts genetically with the core stress signalling pathways and components of stress granules, suggesting a function in stress responses. In support of this, we find that *Drosophila* lacking *CLN3* function are hypersensitive to oxidative stress yet they respond normally to other physiological stresses. Overexpression of *CLN3* is sufficient to confer increased resistance to oxidative stress. We find that *CLN3* mutant flies perceive conditions of increased oxidative stress correctly but are unable to detoxify reactive oxygen species, suggesting that their ability to respond is compromised. Together, our data suggest that the lack of *CLN3* function leads to a failure to manage the response to oxidative stress and this may be the key deficit in JNCL that leads to neuronal degeneration.

INTRODUCTION

Mutations in the *CLN3* gene cause juvenile neuronal ceroid lipofuscinosis (JNCL or Batten disease), a neurodegenerative disorder that affects young people (1). Onset of the disease is usually at 5–7 years, initially with retinal degeneration followed by a progressive loss of neural function resulting in death often by the age of 25 years (2). JNCL is the most common of the NCLs (2), a group of disorders that, with rare exceptions, have an infant or childhood onset, suggesting that the neurodegenerative process is more rapid than in late-onset neurodegenerative diseases. The NCLs share many of the characteristics of the late-onset disorders including localized neural degeneration despite widespread expression of affected genes and generalized accumulation of intracellular deposits (2). The NCLs are characterized by the accumulation of ceroid lipofuscin—autofluorescent storage material—within lysosomes. However, the causes or consequences of this accumulation and its relationship to neuronal degeneration are unclear (3). One possibility suggested by the characteristic early onset of the NCLs is that one or more vital cellular processes are severely or rapidly compromised in the NCLs.

CLN3 encodes a highly hydrophobic, multi-spanning transmembrane protein of unknown function. A variety of approaches have implicated CLN3 in a wide range of cell biological processes, including endocytosis and intracellular protein trafficking (4,5), lysosomal homeostasis (6,7), mitochondrial function (4,8), autophagy (9) and amino acid transport (10). No consensus exists about the function of

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CLN3 nor is there any real understanding of the cellular processes affected by its loss and how these lead to neurodegeneration. In particular, it is not clear which of the affected processes are primary defects and which secondary consequences.

We have employed a complementary approach using *Drosophila* genetics to identify pathways and processes that involve CLN3 function. *Drosophila* provides an excellent model system to study neurodegenerative disease and has a long history as a vehicle to identify the genetic pathways upstream and downstream of known genes (11–16). We adopted a gain-of-function approach to identify pathways and processes that require, or impinge upon, *Drosophila* CLN3 function (15). *CLN3* expression was driven by the UAS–GAL4 system to create a *CLN3*-dependent phenotype, and we subsequently screened for genes that modified this phenotype when co-expressed with *CLN3*. This type of screen functions as a non-biased approach for the discovery of genes that function alongside *CLN3*. One theme emerging from the screen is that *CLN3* interacts with multiple components of stress signalling pathways. We demonstrate that when flies lacking *CLN3* are exposed to oxidative stress, they accumulate reactive oxygen species (ROS) resulting in a hypersensitivity to oxidative stress while responding normally to other physiological stresses. Oxidative stress contributes to neurodegenerative disease but is also a component of the normal ageing process. Our data suggest that in JNCL where ageing is not a significant factor, failure to manage oxidative stress may be fundamental to the disease.

RESULTS

Drosophila contains likely orthologs for 4 of the 10 human genes affected in the NCLs. For the two that have been examined to date (*Ppt1* and *cathepsin D*) (17,18), the core cell biological functions appear to be conserved between mammals and flies. We have used the genetic tools of *Drosophila* to identify genes that modify CLN3 activity. Our aim is to identify genetic interactions for *CLN3* with a view to placing it within a gene regulatory network and to identify the cell biological processes and signalling pathways that require, or impinge upon, CLN3 function.

A non-biased genetic screen for CLN3 interactions

Previously, we have described that increasing the activity of CLN3, but not an inactive form of the protein, in *Drosophila* using the UAS–GAL4 system causes a degenerative phenotype in the eye and induces apoptosis in the wing (15). *CLN3* is expressed in the developing eye but is not required for its development. *CLN3* expression persists in the visual system of the adult fly (Tuxworth and Tear unpublished data) as it does in the mouse (19). To identify potential interacting partners for CLN3, we previously examined candidate genes for their ability to modify the eye and wing phenotypes (15). Here, we have expanded this approach to a larger, unbiased screen to identify further modifiers of the dominant CLN3 phenotypes. Our aim was to identify cell biological processes requiring CLN3 whose specificity could be verified later in *cln3* mutants. A combination of GAL4 drivers and UAS–CLN3 transgenes were chosen that drove *CLN3* expression to a level that causes a phenotype of intermediate severity to allow both enhancers and suppressors to be identified (Fig. 1).

We performed a dominant gain of function screen using 1574 EY P-element insertions on chromosome III. The EY element contains a UAS sequence which allows overexpression of a gene lying close to the insertion site under the spatial control of the chosen GAL4 driver (20). We screened this collection for their effect on our CLN3 phenotypes in both the eye and wing. Subsequently, any elements modifying the phenotypes were discarded if they produced a phenotype in the absence of *CLN3* overexpression, even if the phenotypes were very different. Examination of the genomic location of each modifying element was then used to identify the interacting gene, with *in situ* hybridization used to resolve ambiguities (see Materials and Methods). We identified 7 insertions that enhanced the dominant CLN3 phenotypes in eye and wing, 14 that enhanced the eye phenotype only and 19 that enhanced only the wing phenotype. Only one suppressor of the wing phenotype was identified but 14 suppressors of the eye phenotype (see Supplementary Material, Table S1 for identified genes). Our manual curation of the genes allowed us to assign a significant number of these modifiers to a number of biological pathways or processes.

RNA translational regulators

One cluster of modifiers identified was a group of five RNA-binding proteins (RBPs). RBPs are key components in RNA metabolism regulating all aspects of RNA biogenesis from RNA splicing, maturation and transport to RNA translation. Many RBPs have been identified as being involved in human disease, principally neurodegenerative disorders (21). Of these, Boule and Pumilio are known to be translational repressors with functions in the nervous system (22–24). Both showed strong interactions with CLN3 in this screen (Fig. 1C and G for Boule, Pumilio not shown). We also identified *pumilio* in a further genetic screen using chromosomal deficiencies to identify genomic regions that harbour loci that when present in a single copy modify a CLN3 gain-of-function phenotype (15) (Tuxworth and Vivancos, unpublished data). Rox-8, an RBP with strong sequence homology to the mammalian TIA-1 protein (25), also shows strong interactions with CLN3 (Supplementary Material, Table S1). Previously, we had identified interactions with mago-nashi and Y14/tsunagi, two components of the RNA-binding exon junction complex (EJC) (20). The EJC has a major role in post-transcriptional regulation of mRNA translation and localization (26–28). In this screen, it is unlikely that *CLN3* expression levels are affected by co-expression of these regulators since the transgenes used carry only the *CLN3* ORF with no endogenous *CLN3* 5′ or 3′ UTR sequences and there is no requirement for RNA splicing. There does appear, however, to be a role for post-transcriptional regulators in modifying CLN3 function. A common function of both TIA-1, pumilio-2 and the MLN51 component of the EJC is their

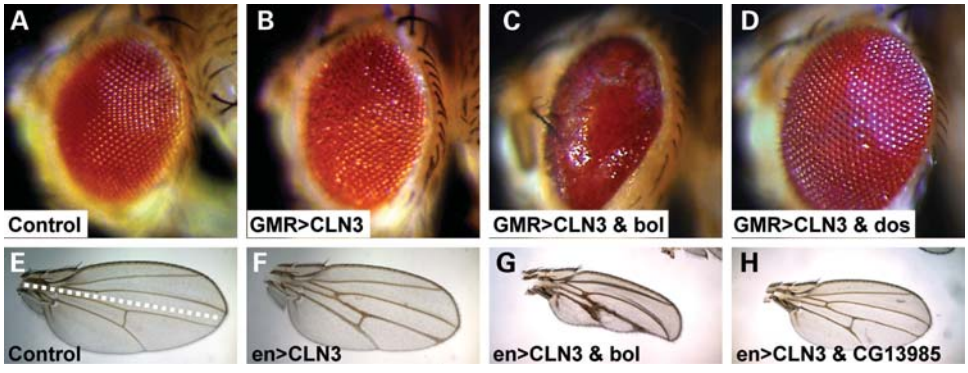

Figure 1. A screen for genetic interactions with CLN3. A collection of EY insertions was used to mis-express genes in the eye and wing to modify the phenotypes resulting from overexpression of *CLN3*. (**A**–**D**) *GMRgal4* was used to overexpress *CLN3* in the developing eye. This results in roughening and glazing of the outer surface in adult flies (B, compare with driver control in A). Both enhancers (C) and suppressors (D) of the original phenotype were detected. (**E**–**H**) *Engrailed-gal4* was used to overexpress *CLN3* in the posterior half of the wing. This results in thickened veins, loss of tissue, vein deltas and occasionally notching in the wing margin (F, compare posterior below the dashed line with anterior above and with driver control in E). Several enhancers (G) and one suppressor (H) were identified.

recruitment to cytosolic stress granules–aggregates of transiently silenced mRNAs that form after exposure of cells to environmental stress (25,29,30).

CLN3 interacts with components of stress signalling pathways

A major group of modifiers identified in the screen are known to be involved in stress response signalling; Mekk1 is a MAPKKK that activates the p38 MAP kinase in *Drosophila* in response to environmental stress (31); l(3)82FD is the *Drosophila* OXR1 protein required for oxidation resistance in yeast and mosquitoes (32); JafRac2 has similarity to thioredoxin peroxidases, a family of proteins that protects against protein peroxidation; and falafel is a subunit of the PP4A phosphatase complex that is required for some stress response signalling in *C. elegans* (33). These interactions, together with a potential link to stress granules and with our previous finding that CLN3 can ectopically activate JNK signalling (15), which is central to stress responses and neuropathology (34), suggest that CLN3 may be involved in the cellular response to stress.

Dominant *CLN3* phenotypes are mediated by stress response pathways

We investigated further whether the *CLN3* overexpression phenotype in the eye is modified by key regulators of the stress response. The forkhead transcription factor, Foxo, integrates antagonistic signalling from the nutrient-sensing insulin-signalling and stress-induced JNK signalling pathways (35,36). We combined UAS–CLN3 with UAS–Foxo to overexpress both proteins in the developing eye. Overexpression of *foxo* alone produces a small patch of severe glazing in each eye, presumably due to alterations in cell size within the developing eye (Fig. 2B). The addition of CLN3 produces a much wider glazing of the eye indicating a strong genetic interaction (Fig. 2C) and suggesting that CLN3 may act within a stress response pathway. Consistent with this, the dominant CLN3 phenotype is partially suppressed by reducing the *foxo* gene dosage with the $foxo^{21}$ allele (Fig. 2D). Further support for a role for CLN3 in the Foxo pathway was provided by the identification of *falafel* as a suppressor in our screen. Falafel is a regulatory subunit of the PP4A phosphatase and in *C. elegans*, the falafel homologue, *smk-1,* acts downstream of *daf-16* (the *foxo* homolog) in some stress signalling responses, including oxidative stress (33). Co-expression of Falafel from an independent UAS transgene almost completely suppresses the *CLN3* overexpression phenotype (Fig. 2E).

We also examined whether CLN3 interacts with a second signalling pathway central to cellular stress responses—the keap1/Nrf2 pathway—that has also been implicated in neurodegenerative disease (37). We find that reducing the dosage of *keap1* (the cytosolic inhibitor of the Nrf2 transcription factor) gene almost completely suppresses the dominant *CLN3* eye phenotype (Fig. 2F). Since heterozygosity at the *keap1* locus confers stress resistance to flies and extends lifespan (38), we wondered whether increasing CLN3 levels in the eye was affecting the oxidative stress response, perhaps through dominant interference. We used a second method of increasing oxidative stress resistance in the developing eye by overexpressing the ROS scavengers $Cu^{2+}Zn^{2+}$ superoxide dismutase or catalase alongside CLN3. Both led to almost complete suppression of the phenotype (Fig. 2G and H) strongly suggesting that CLN3 is in some manner affecting the oxidative stress response. Given that oxidative stress is implicated as a major factor in a number of neurodegenerative diseases, we determined the relevance of these findings by asking whether the absence of CLN3 might impair the ability of animals to manage oxidative stress.

Generation of a *cln3* mutant

JNCL results from a loss of CLN3 activity. To investigate further a potential role for CLN3 in stress responses, a mutation in the *CLN3* locus was created by imprecise excision of a Minos transposable element (39) inserted within the large first intron. The excision deletes 1534 bp including the predicted translation start site and the first 102 codons of CLN3 (see Supplementary Material, Fig. S1) and is therefore likely

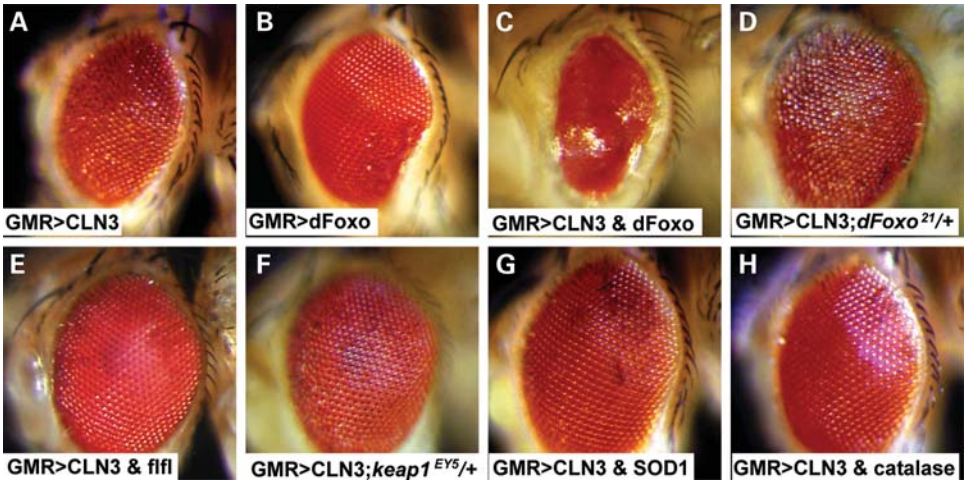

Figure 2. *CLN3* interacts genetically with components of stress signalling and effectors of the oxidative stress response. (**A**–**E**) The *CLN3* overexpression phenotype in the eye is dependent on the levels of the dFoxo protein and dFoxo signalling. Co-overexpression of *CLN3* and *dFoxo* (C) results in a much more severe phenotype than either (A) *CLN3* or (B) *dFoxo* alone. (D) A heterozygous $dFoxo^{21}$ background results in partial suppression of the phenotype. (E) Co-overexpression of *Drosophila falafel* and *CLN3* completely suppresses the phenotype. The *C. elegans* homologue of *falafel*, *smk-1*, mediates Foxo signalling in the oxidative stress response. (**F**) Reduction in *keap1* gene dosage leads to stabilization of the Nrf2 transcription factor that mediates antioxidant effector gene expression. In *keap1* heterozygous flies, the *CLN3* overexpression phenotype is suppressed completely. (**G** and **H**) Overexpressing the scavengers $Cu^{2+}Zn^{2+}$ *superoxide dismutase* (G) or *catalase* (H) in the eye suppressed the *CLN3* overexpression phenotype completely.

to be a functional null. An antiserum raised to the N-terminus of *Drosophila* CLN3 (15) fails to detect the CLN3 protein in homozygous mutant animals. We have named the mutant allele $cln3^{\Delta MB1}$. The mutant flies are viable and fertile with no obvious developmental abnormalities. The viability of mutant embryos is reduced by a small degree but survival of first instar larvae to adulthood is unaffected (data not shown). The lack of an overt external phenotype is similar to that observed for mouse mutants of CLN3 (40,41) and for other NCL mutations, including the *Drosophila* mutants in Ppt1 (18) and cathepsin D (17). Our initial observations indicate no accumulation of autofluorescent material in the brain of aged $cln3^{\Delta MB1}$ flies.

cln3 mutant flies are hypersensitive to oxidative stress

We tested the susceptibility of the *cln3* mutant animals to oxidative stress induced by three different oxidants: hydrogen peroxide which generates hydroxyl radicals via the Fenton reaction, diethylmaleate (DEM) which depletes cellular stores of glutathione and paraquat which generates superoxide anions. Survival of the *cln3* mutant flies was compared with that of an appropriately matched isogenic control strain. In all three cases, the *cln3* mutant flies were hypersensitive to oxidative stress when compared with control animals (Fig. 3A–C). To confirm that the phenotype is caused solely by the loss of CLN3 activity, we re-expressed *CLN3* in the mutant flies. Ubiquitous expression of a *Venus-CLN3* cDNA rescues the sensitivity of mutant flies to paraquat (Fig. 3C). Consistent with this hypersensitivity to oxidative stress, the *cln3* mutant flies also show a significantly reduced lifespan that is also rescued by expression of a *Venus-CLN3* cDNA (Fig. 3D).

CLN3 is specific to the oxidative stress response

We also investigated whether the hypersensitivity of *cln3* mutant flies to oxidative stress reflects a non-specific sensitivity to all environmental stresses, perhaps due to a role for CLN3 in a more general physiological process. We tested the sensitivity of the mutant flies to thermal stress by measuring the time for recovery from cold-induced coma (Fig. 3G) and survival times at 37°C. No changes in response were seen between control and mutant flies. We also tested the resistance of flies to starvation (Fig. 3H) and developmental viability under increasing osmotic stress (data not shown). In neither case were *cln3* mutant flies more susceptible to the stress. Indeed, *cln3* mutant female flies are significantly more resistance to starvation than wild-type flies (Fig. 3H), although males show no difference (data not shown). Clearly, loss of CLN3 activity does not lead to a general reduction in health of flies, rather CLN3 appears to be specifically required for the oxidative stress response.

Neural expression of *CLN3* confers resistance to oxidative stress

We next asked whether increasing *CLN3* expression in wild-type flies is sufficient to confer an increased resistance to oxidative damage. Activation of JNK signalling specifically in the insulin-secreting neurons (Dilp neurons) of the *Drosophila* brain is sufficient to confer an animal-wide resistance to stress and to extend lifespan via an inhibition of insulin secretion (42). We have reported previously that overexpression of *CLN3* in the wing results in an ectopic activation of JNK signalling. Hence, we reasoned that ectopic expression of *CLN3* in the Dilp neurons may similarly activate JNK signalling and so confer increased stress resistance to the whole fly and extend lifespan. Using the Dilp2–Gal4 driver line

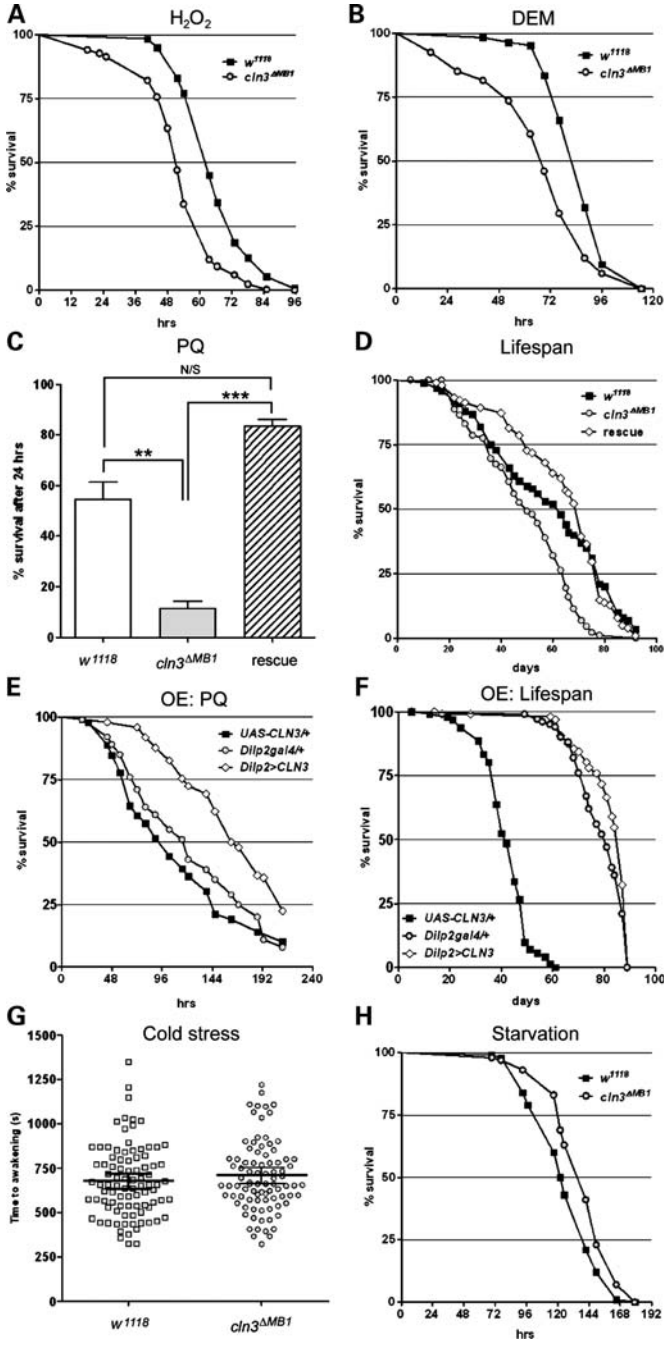

Figure 3. *Cln3* mutant flies are hypersensitive to oxidative stress. (**A**–**D**) Isogenic control (w^{1118}) and $cln3^{\Delta MB1}$ flies were exposed to oxidants via feeding. (A and B) Survival analysis for flies exposed to (A) hydrogen peroxide or (B) DEM indicates that the mutants are significantly hypersensitive to either oxidant (log-rank analysis $P < 0.001$ in both cases). (C) 24 h exposure to 20 mM paraquat results in ~50% lethality in control flies (white bar), while $cln3^{\Delta MB1}$ flies are hypersensitive (grey bar) and display greater lethality. Ubiquitous expression of a *VenusCLN3* cDNA in the $cln3^{\Delta MB1}$ flies completely rescues the hypersensitivity (hashed bar). Error shown is SEM. $^{***}P < 0.001$, $^{**}P < 0.01$, N/S $P > 0.05$. (D) Hypersensitivity to oxidative stress results in a reduced median lifespan of $cln3^{\Delta MB1}$ flies under normal culturing conditions which are rescued by ubiquitous expression of VenusCLN3 in the $cln3^{\Delta MB1}$ flies. Median lifespan: w^{1118} 63 days versus $cln3^{\Delta MB1}$ 50 days ($P = 0.003$), w^{1118} versus *arm > VenusCLN3;* $cln3^{\Delta MB1}$ 71 days *(N/S)*. (**E** and **F**) In a wild-type background, overexpression of *CLN3* in Dilp-secreting neurons is sufficient to confer increased resistance to paraquat and extend lifespan. (E) Flies were exposed to low levels of paraquat for several days. Flies overexpressing *CLN3* (diamonds) were more resistant than either control line. Median survival: *UAS–CLN3/+* 103 h, *Dilp2–gal4/+* 121 h, *Dilp2 > CLN3* 165.5 h. (Overexpression versus Gal4 control $P < 0.001$). (F) A small but significant extension to median lifespan is seen in comparison to controls. Median lifespan: *UAS–CLN3/+* 42 days, *Dilp2–gal4/+* 81 days, *Dilp2 > CLN3* 87 days. Overexpression versus Gal4 control $P = 0.009$). (**G** and **H**) *CLN3* is not required for all stress responses. (G) No difference was detected in the time taken for flies to recover from cold-induced coma. Median recovery time: w^{1118} 657 s, $cln3^{\Delta MB1}$ 660 s. Mean and 95% confidence intervals are indicated for 90 observations per genotype. (H) Survival analysis for starved flies indicates a small but significant increase in resistance of female $cln3^{\Delta MB1}$ flies in comparison to controls. Median survival: w^{1118} 124 h, $cln3^{\Delta MB1}$ 142 h, $P < 0.001$). No differences were detected in males.

(43), we expressed *CLN3* in these neurons in a wild-type background and tested sensitivity to paraquat over several days (Fig. 3E) and also measured lifespan under normal rearing conditions (Fig. 3F). The UAS–CLN3 insertion itself had the effect of reducing both paraquat resistance and lifespan (Fig. 3E and F and compare with the median lifespan of 63 days for the w^{1118} control strain in Fig. 3D). In contrast, flies heterozygote for the Dilp2–gal4 driver alone extended lifespan (Fig. 3E and F). As predicted, when the Dilp2–Gal4 and UAS–CLN3 insertions were combined to overexpress *CLN3* in the Dilp-secreting neurons, these flies were significantly more resistant to paraquat than either control line and showed a small but significant extension of lifespan (Fig. 3E and F).

ROS accumulate in *cln3* mutant flies

The *cln3* mutant flies may be hypersensitive to oxidative stress due to an increased rate of ROS production, a failure to perceive the presence of ROS or due to a failure to mount an effective response to ROS. To quantify ROS in flies, we used a common indicator of peroxide activity, the cell-permeant reporter, CM-H_2DCFDA, that is cleaved by cellular esterases and becomes fluorescent upon oxidation by cellular ROS (44). Control flies administered H_2O_2 show very little increase in ROS levels, presumably reflecting upregulation of antioxidant defences (Fig. 4A). *cln3* mutant flies have a small but not statistically significant increase in ROS levels but a dramatic increase when H_2O_2 is administered (Fig. 4A). Seemingly, the mutant flies are unable to detoxify the increased ROS that accumulates when exposed to conditions of oxidative stress.

The transcription response to oxidants is intact in *cln3* mutant flies

We asked whether the accumulation of ROS in the *cln3* mutant flies was due to a failure to upregulate antioxidant gene transcription in response to oxidants. We used a genetically encoded reporter, *gst::GFP*, which contains antioxidant response elements sequences from the *gstD* promoter upstream of a *GFP* cDNA (38). *GFP* expression is induced in response to multiple oxidants via the activity of the Nrf2 transcription factor (38). We asked whether the *GFP* reporter is induced correctly in the *cln3* mutant flies. In the absence of oxidants, the reporter is expressed predominantly in the

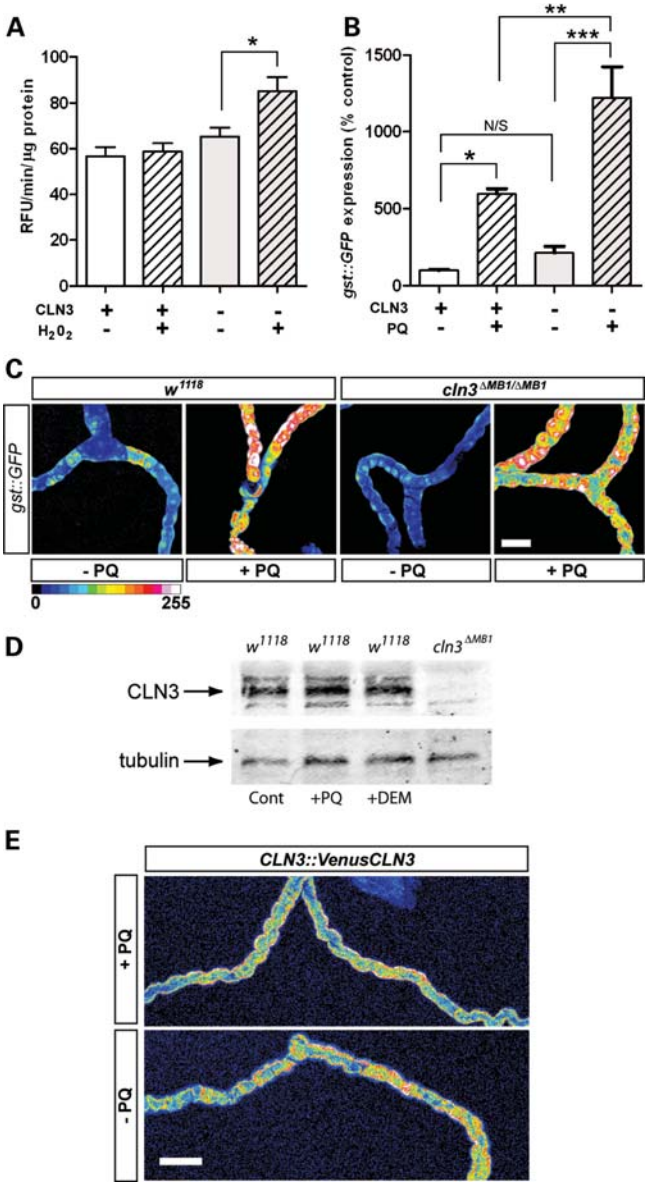

Figure 4. *cln3* mutant flies respond to oxidants but fail to remove ROS. (**A**). w^{1118} and $cln3^{\Delta MB1}$ flies were administered H_2O_2 for 60 h before ROS levels were quantified in tissue homogenates using a fluorescent reporter, DCF (44). Wild-type flies show no significant increase in ROS levels following administration of H_2O_2. Mutant flies display a small but insignificant increase in ROS levels in the absence of oxidant. Following H_2O_2 administration, ROS levels increase significantly in the mutant flies indicating a likely failure in detoxification of the oxidant. Error shown is SEM. $^{*}P < 0.05$. (**B**–**C**) $cln3^{\Delta MB1}$ flies show a robust transcriptional response to oxidants. A *gst::GFP* reporter was introduced to control w^{1118} and $cln3^{\Delta MB1}$ flies. The reporter expresses GFP and is induced by a wide range of oxidants. (B) Quantitative western blotting was used to measure GFP levels in adult flies normalized to that of control flies under non-insulted conditions. Induction is seen in both control and $cln3^{\Delta MB1}$ flies after administration of paraquat. The starting level of GFP expression is slightly higher in $cln3^{\Delta MB1}$ flies and the degree of induction is much greater. Error shown is SEM. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, N/S $P > 0.05$. (C) The reporter is expressed mainly in the Malpighian tubules and is highly induced in response to oxidant insult. Tubules from w^{1118} and $cln3^{\Delta MB1}$ flies were dissected following paraquat administration and imaged live using identical settings. $cln3^{\Delta MB1}$ tubules respond similarly to wild-type. Images are displayed with a 4-bit colour scale to show induction qualitatively. The colour scale is displayed below. Bar = 5 µm. (**D** and **E**). *CLN3* expression is not induced in response to oxidants. (D) A western blot of whole fly lysates indicates no induction in CLN3 protein levels in response to paraquat or DEM administration. Tubulin is used as a loading control. (E) *CLN3* is highly expressed in the Malpighian tubules and is not induced in tubules in response to oxidants. VenusCLN3 under the control of the endogenous *CLN3* promoter was imaged live from dissected tubules with or without paraquat exposure. Images are displayed with the same 4-bit colour scale as in (C). Bar = 5 µm.

Malpighian tubules, an organ with an analogous physiological function to the mammalian kidney and reflecting the importance of the tubules in detoxification (Fig. 4C). The basal level of GFP expression is ~2-fold higher in the mutant flies, indicating that they are under an intrinsic oxidative load (Fig. 4B) and consistent with the small increase in ROS levels in mutant flies under basal conditions (Fig. 4A). This increase is small but consistent and may relate to an increase in ROS production in the mutant flies or a failure in the effector response. It is also consistent with previous findings from patient-derived neuronal cultures (45). Following exposure to either paraquat or DEM, the reporter was induced strongly in the tubules of both wild-type and *cln3* mutant flies (Fig. 4C). In fact, mutant flies induce the reporter to a higher level (Fig. 4B). Thus, CLN3 is not required for perception of oxidative stress and the major transcriptional response remains functional in its absence.

We asked whether CLN3 could itself be directly involved in the antioxidant effector response. If so, we would expect CLN3 levels to be upregulated in response to oxidants, as is true for many antioxidant effectors. *Drosophila CLN3* is highly expressed in the Malpighian tubules (Fig. 4E and Tuxworth, unpublished data) consistent with the observation that murine *CLN3* is similarly expressed in the kidney (46) and perhaps reflecting a role in detoxification. An antibody to CLN3 (15) showed no increase in protein levels in whole fly lysates exposed to either paraquat or DEM (Fig. 4D). However, since CLN3 has a restricted expression in adult flies, it seemed possible that induction of *CLN3* in tubules would not be detectable using whole fly lysates. To address this, we examined the induction of a *Venus-CLN3* construct directly in live tubules. This fusion is expressed at endogenous levels from the endogenous *CLN3* promoter. Again, no increase in the CLN3 protein levels were seen following exposure to paraquat (Fig. 4E). It seems unlikely, therefore, that CLN3 is a direct effector of the antioxidant response but provides a constituent activity that is necessary to mount a response to increased levels of oxidative stress.

DISCUSSION

Mutations in the human *CLN3* gene give rise to JNCL, the most common variant of the NCLs (1,2). Currently, the function of CLN3 is not clearly defined nor is it understood how loss of CLN3 function leads to pathogenesis (47,48). Here we used a dominant gain-of-function modifier screen in *Drosophila* as a discovery tool to identify genes that interact with *CLN3* and indicate which primary cellular pathways might be affected by its loss. *Drosophila* contains homologues of several of the genes affected in the NCLs, suggesting that the functional pathways that utilize these genes are likely to be conserved (15,17,18,49). We identified interactions between *Drosophila CLN3* and components of stress signalling

pathways and RBPs found in stress granules. These interactions suggest that CLN3 might play a role in stress signalling. To validate these findings and determine their relevance for JNCL, we generated a *CLN3* mutant to model the loss-of-function disease and find that the mutants have a specific requirement for CLN3 to respond to oxidative stress. This allows us to speculate that the neural degeneration that occurs in the disease may be a consequence of an inability of neurons to manage oxidative stress.

Oxidative stress, juvenile NCL and lysosomal dysfunction

The precise cellular functions of CLN3 within the nervous system are unknown. JNCL patients and mouse *Cln3* mutants display lipofuscin accumulation and lysosomal dysfunction. Previous studies have suggested roles for CLN3 in intracellular protein trafficking and endocytosis (4,5), lysosomal homeostasis (6,7), autophagy (9), mitochondrial function (4,8), amino acid transport (10) or regulation of the actin cytoskeleton (50,51) and that failures in any of these roles may lead to the disease pathology. However, it is not yet known which of these roles are the key *in vivo* neural functions of CLN3 or those that are impaired as a secondary consequence of the lack of CLN3 activity. In common with other neurodegenerative diseases, it is difficult to disentangle which elements of the observed pathology are causes or consequences of the primary molecular defect, making it challenging to identify the initiating events that lead to disease. Here we used complementary strategies: a gain-of-function approach to identify the pathways and processes that impinge upon CLN3 function and use of loss-of-function to verify their role. Our observations suggest a potential protective role for CLN3 in managing the cellular response to oxidative stress. A lack of CLN3 leads to an accumulation of ROS and this accumulation will lead to increase in oxidized cytosolic proteins that could lead to lysosomal dysfunction and cell death. A potential role for oxidative damage in the disease has also been highlighted in a mouse model of JNCL (52) and is suggested by an increased sensitivity to hydrogen peroxide of a cerebellar cell line derived from *Cln3* mice (4).

Evidence is accumulating to suggest that oxidative damage contributes significantly to many neurodegenerative disorders (53,54). Accumulation of oxidized proteins and lipids affects mitochondrial and lysosomal function resulting eventually in cellular death. Neurons appear to be particularly susceptible to oxidative damage potentially due to a combination of their longevity and high metabolic demand resulting in an increased oxidative load (55). However, determining whether oxidative damage is pivotal to neurodegenerative disease progression is difficult, one complication being the inability to dissociate completely the disease process in late-onset disorders from the normal ageing process. By studying JNCL, a neurodegenerative disease with childhood onset, it may be possible to determine the relative importance of oxidative stress in the disease process. Our findings highlight a specific role for CLN3 in the oxidative stress response and suggest that a compromise of this response may be a fundamental defect leading to neurodegeneration in JNCL.

There is some histological evidence from *Cln3* and *Cln8* mouse mutants that the balance between pro- and anti-oxidant molecules in brain sections changes with disease progression (52,56). Additionally, the levels of the anti-oxidant scavenger protein MnSOD are elevated in brain material from both *Cln3* mutant mice (52) and *Cln6* mutant sheep (57). These changes may be secondary to the primary cause of neurodegeneration, but the regions affected do correlate with the neuronal loss. Our data suggest that the principal defect in the disease could be the inability to respond appropriately to oxidative stress. Lipofuscin accumulation and lysosomal dysfunction are known consequences of oxidative damage (58). This accumulation stems from the resistance of cytosolic oxidized proteins to lysosomal degradation and an increase in intralysosomal hydroxyl radicals levels. Similarly, endoplasmic reticulum and mitochondrial function are impaired by oxidative stress, generating further oxidative load upon cells. It is interesting to note that the oxidative load is increased in neurons cultured from various NCLs, including JNCL (45,59), and in our *cln3* mutant flies.

The role of CLN3 in the oxidative stress response

How does CLN3 function in the response to oxidative stress? Flies lacking *cln3* accumulate ROS when challenged (Fig. 4A) yet induce a GST-based reporter correctly (Fig. 4B and C), indicating that they remain capable of perceiving the presence of the damaging oxidant and that the signal transduction pathway leading from perception to induction of the transcriptional response to oxidative stress is intact. In fact, the mutant flies appear to hyper-induce this reporter which may reflect a feedback mechanism where expression of effectors is elevated as cells fail to combat oxidative damage.

Could CLN3 be involved directly in the defense against ROS as a scavenger or antioxidant? Both paraquat and hydrogen peroxide are direct sources of ROS, whereas DEM indirectly sensitizes cells to endogenously produced ROS by depleting glutathione, the major cellular substrate used for ROS reduction. If CLN3 was directly involved in combating a particular class of ROS, then its loss might sensitize cells to the presence of additional ROS or to a reduction in glutathione, however generated. Arguing against this is the failure of flies to upregulate *CLN3* transcription in response to oxidants (Fig. 4D and E). We have examined both CLN3 protein levels and transcription of a CLN3 fusion protein expressed from the *CLN3* promoter finding no evidence for induction. We suggest that CLN3 may function as a constituently expressed component of a process necessary for an effective response to increased levels of oxidative stress.

The expression of the *gst::GFP* reporter expression is increased in the *cln3* mutant flies under basal conditions (Fig. 4B). The increase is consistent but small and falls below the threshold of statistical significance but might indicate a small increase in the endogenous oxidative load. Previous reports have shown such an increase in the endogenous stress load in primary neuronal cultures from several NCL mouse models. In this case, the buffer of antioxidant defence may be depleted in *cln3* mutant flies leading to enhanced oxidative damage accumulation from exogenously applied oxidants or the ageing process. This accumulation

may initiate the cascade of events which result in defects in lysosomal, mitochondrial and autophagic function observed in models of JNCL.

CLN3 and stress signalling

Central to stress signalling in eukaryotes are the JNK MAP kinase signalling pathway and the forkhead transcription factor, Foxo (36). Foxo integrates signalling from the JNK pathway that promotes stress resistance and the insulin signalling pathway that plays an antagonistic role in promoting growth and reproduction. CLN3 displays genetic interactions with both JNK and Foxo, and overexpression ectopically activates the JNK pathway. However, both JNK and Foxo are required for most environmental stress responses, whereas CLN3 appears specific to oxidative stress. It is possible that CLN3 is required to activate JNK in response to the presence of oxidants. Alternatively, CLN3 may lay downstream of Foxo in this pathway and the ectopic activation of JNK following *CLN3* overexpression is due to some feedback loop.

We identified interactions with falafel, a component of the PP4A complex and homologue of *C. elegans smk-1* (33). In *C. elegans*, *smk-1* mediates Foxo signalling (daf-16) in the oxidative stress response but is not required for the heat stress response (33). This mirrors our results for *cln3* mutant flies and it may reflect a similar uncoupling of the stress signalling downstream of *Drosophila* dFoxo. An epistasis-style analysis with JNK, dFoxo and falafel and also with the keap1/Nrf2 pathway will allow CLN3 to be placed within the known stress signalling pathways.

MATERIALS AND METHODS

Drosophila husbandry and stocks

Flies were reared at low density in bottles at 25°C with a 12 h light/dark cycle on cornmeal-based medium except for the lifespan analysis and osmotic stress assays when SY food was used (60). Media recipes are available from the Bloomington stock centre website.

For all assays, flies eclosing over a 24 h period were transferred to fresh culture bottles and allowed to breed for 24–48 h before being separated by sex. Assays were performed only with female flies. The control strain used for all experiments was an isogenic w^{1118} line (Vienna Drosophila RNAi Center). For stress assays and lifespan analysis, all lines used were backcrossed for six generations to w^{1118}. Armadillo-gal4 and Dilp2–gal4 were used to drive UAS–CLN3 or UAS–VenusCLN3 expression ubiquitously or in insulin-secreting neurons, respectively, and are described in Flybase. UAS–CLN3 and UAS–VenusCLN3 were cloned using pTW and pTVW, respectively (Murphy collection available from Drosophila Genomics Resource Center, Indiana). Lines were generated by standard P-element-mediated germline transformation.

Generation of a *cln3* mutation

The MB6009 Minos insertion in the *CLN3* locus (39) was excised with the Minos transpose using a standard crossing scheme. Excision chromosomes were screened for deletion events by PCR. The $cln3^{\Delta MB1}$ allele generated was backcrossed six times to the isogenic w^{1118} control strain before use.

Mis-expression screen

Overexpression of *CLN3* in the eye and wing was as previously reported (15). 1574 EY elements inserted on chromosome III were supplied by the Bloomington stock centre. Progeny carrying the driver, UAS–CLN3 and EY element were scored for modification of the original *CLN3* overexpression phenotype. EY elements producing phenotypes in the absence of UAS–CLN3 were discarded. Scoring of the strength of interaction was qualitative, based on multiple repetitions. *In situ* hybridization by standard methodology was used to identify which gene(s) was being mis-expressed from an EY element when the genome annotation was ambiguous.

Oxidative stress assays

For hydrogen peroxide, DEM and the long-term paraquat assays, drugs were added to a melted 1.3% w/v low melting point (LMP) agarose, 5% w/v sucrose solution at 45°C before 2.5 ml aliquots were dispensed into plastic vials. DEM was diluted first to 1 M in ethanol before a second dilution in the agarose solution. Final concentrations were 1% H_2O_2, 10 mM DEM and 11.5 mM paraquat. Five cohorts of 20 female flies were tested in each case and deaths recorded two or three times per day. All experiments were performed in the dark.

For short-term paraquat assays, 15 cohorts of 10 female flies were dehydrated in empty vials at 25°C for 3–4 h then transferred to vials containing a 15 × 15 mm square of thick filter paper soaked in a solution of 20 mM paraquat in 5% w/v sucrose. All assays were started between 16:00 and 17:00 h to avoid variation between experiments due to circadian rhythms. Deaths were recorded at 18, 21 and 24 h.

Thermal stress assays

For heat stress, female flies were transferred in cohorts of 5 to vials containing 1.3% w/v LMP agarose in 5% w/v sucrose. Vials were submerged to a level just below the flug at 37°C and deaths events scored every 5 min. A death event was considered to be complete paralysis. For cold stress, female flies were placed in cohorts of 3 in glass vials and placed on wet ice for 4 h to induce cold coma. Vials were transferred to room temperature and the time to recovery from coma recorded. Recovery was considered to be a fly capable of standing upright.

Lifespan assays

Zero to 24 h old flies were allowed to breed for 24 h, and then transferred to vials of SY food in cohorts of 10. One hundred female flies were used for each genotype. Flies were transferred to fresh vials three times per week and deaths recorded.

Quantification of ROS

One- to 3-day-old flies were transferred in cohorts of 25 to agarose/sucrose vials with or without H_2O_2 at 3% final concentration. After 60 h, 10 flies from each vial were homogenized in 500 μl ice-cold 10 μM CM-H_2DCFDA (Invitrogen) solution in PBS. Homogenates were centrifuged for 5 min at 14 000 rpm at 4°C. One hundred microlitre lysate was then transferred to a black 96-well plate in triplicate and the fluorescence read every 5 min for 3 h at 27°C in a Flexstation fluorimeter (Molecular Devices) using Ex 485 nm, Em 538 nm and cut-off 530 nm. The steady state rate of fluorescence accumulation in each lysate was normalized to the protein concentration determined by the Bradford assay. PBS was made using HPLC-grade water. CM-H_2DCFDA was dissolved in anhydrous DMSO at 10 mM immediately before dilution in PBS.

Quantitative western blotting

One- to 2-day-old female flies were transferred to SY food containing oxidants as above. After 36 h exposure, groups of 5 flies were transferred to 2.0 ml tubes containing garnet beads and homogenized in a FastPrep homogenizer (MP Bioscience) twice for 15 s at 6.0 m/s in 150 μl of lysis buffer (50 mM Tris, pH 7.5; 150 mM NaCl; 1 mM NaEGTA; 1 mM NaEDTA) supplemented with a protease inhibitor cocktail (Calbiochem). SDS lysis buffer was then added, samples boiled for 5 min and loaded immediately onto gels. SDS–PAGE, blotting and immunodetection were all by standard methodology. Anti-GFP IgG (Roche), anti-CLN3 (15) and anti-actin IgM (DSHB, University of Iowa, IA, USA) were used to quantify GFP levels normalized to total actin using an Odyssey infrared scanner (Licor). Samples were run five times.

Quantitation of expression levels in Malpighian tubules

One- to 2-day-old female flies of genotypes w^{1118};*gst::GFP/+*;+ and w^{1118};*gstD::GFP/+*;*cln3*$^{\Delta MBT}$ were allowed to lay on grape juice plates for 4–6 h and the embryos then developed for 24 h at 25°C. First instar larvae were then transferred to vials containing SY food supplemented with 10 mM paraquat or 5 mM DEM with 100 larvae per vial. Malpighian tubules were dissected in HL3 buffer (61) from the first wandering third instar larvae emerging from the food. GFP fluorescence in tubules was then visualized immediately on a Zeiss 510 confocal microscope using a narrow band-pass filter to eliminate autofluorescence. Tubules exposed to oxidative stress were visualized first to establish appropriate laser power and gain settings. Subsequently, non-treated tubules were visualized with identical settings. Eight-bit images were recorded and displayed in a 4-bit colour scale.

To visualize CLN3 expression levels, flies were generated that carry a BAC (62) engineered by recombineering (63) to express *VenusCLN3* under the control of the endogenous *CLN3* promoter. The *VenusCLN3* expression pattern and subcellular localization of the fusion protein completely matches that seen previously using an antibody to endogenous CLN3. These flies will be described more fully elsewhere. Expression levels in tubules were imaged as for the *gst::GFP* lines.

Statistical analysis

All analysis was performed in the GraphPad Prism package. For oxidative stress tests performed in agarose, lifespan analysis, heat shock and starvation tests, log-rank tests were used to determine statistical significance. Kruskal–Wallis tests were used for short-term paraquat resistance experiments, Chi-squared analysis for cold stress experiments and ANOVA for western blots and ROS quantification.

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Conflict of Interest statement. None declared.

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