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**Evidence that mutations in the CLN3 gene cause failed oxidative stress response and contribute to Batten disease in *Drosophila***

Objectives:

Neuronal ceroid lipofuscinosis disorders (NCLs) are a group of neurodegenerative diseases that typically have early childhood onset. Juvenile neuronal ceroid lipofuscinosis (JNCL or Batten disease) is the most common NCL and is caused by single gene mutations in the *CLN3* gene. The CLN3 gene encodes a highly hydrophobic transmembrane protein, and there has been much speculation that this protein is involved in important cellular processes such as endocytosis, mitochondrial function and amino acid transport. Currently, the function of the CLN3 gene protein is unknown and it is also unknown which cellular pathways and processes are affected by the expression of the CLN3 gene protein. Studying the function of the CLN3 gene protein is important because reduction in CLN3 gene expression causes symptoms seen in Batten disease, including macular degeneration and early fatalities in humans. Understanding the function of the target protein will allow insight as to how mutations in the CLN3 gene result in neuronal degradation. The main goal of Tuxworth et al. was to identify cellular pathways that involve the CLN3 gene using a *Drosophila* model system. *Drosophila* are used in this study because their genome contains likely orthologs for 4 of the 10 human genes associated with NCLs. CLN3 is among the 2 likely orthologs that has not been intensely studied for function. Tuxworth et al. aim to discover the role of CLN3 and determine how CLN3 mutations cause Batten disease and neuronal degradation.

Experimental Approach and Results:

In previous studies by Tuxworth et al. it was determined that an inactive CLN3 protein in *Drosophila* caused degenerative eye and apoptosis wing phenotypes. Tuxworth et al. (2011) performed a non-biased genetic screen for CLN3 mutations to determine candidate genes that caused phenotypes in the eye and wing of *Drosophila*. The goal of this screen was to identify genes that might be acted upon (or required) by the CLN3 gene protein. A UAS-GAL4 system was used to derive a transgene that caused an intermediate severity phenotype, so that enhancers and suppressors of the phenotype could be scored for modification and strength of interaction with CLN3. A dominant gain of function screen was performed using 1574 EY P-element insertions on chromosome 3. The transposon insertions that caused a noticeable phenotypic modification in *Drosophila* were scored as either suppression or enhancement elements. *In situ* hybridization was used to determine the genomic location of the gene affected by the transposon insertion. Only elements causing phenotypic modification in the presence of CLN3 overexpression were considered in the results. Tuxworth et al. identified 7 insertions that enhanced both wing and eye phenotypes, 14 that enhanced eye phenotype only, and 19 that enhanced wing phenotype only. 14 suppressors of eye phenotype and 1 suppressor of wing phenotype were also found. A cluster of modifiers that interact with the CLN3 gene product were identified and characterized as RNA binding proteins (RBPs).RNA translational regulators, such as these, play an important role in RNA maturation, including RNA splicing and transport to RNA translation. RBP Boule is known to be a translational repressor of the nervous system, and showed intense association with CLN3 (Fig. 1C and 1G for Boule). Noteworthy Rox-8 (an RNA-binding protein) showed an intense interaction with CLN3. The likely ortholog to Rox-8 in humans, TUA-1 protein, and pumilio-2 have a function in recruitment of cytosolic stress granules – elements that form after exposure of cells to environmental stress. Tuxworth et al. found that a large group of identified modifiers from their screen are known for their involvement in signaling a cellular stress response. These modifiers include: Mekk1, 1(3)82FD, JafRac2, and falafel – elements all known to signal a stress response in *Drosophila* and who interact with CLN3.

In order to study the possibility that CLN3 is involved in stress signaling responses, Tuxworth et al. investigated whether regulators of the cellular stress response would modify the CLN3 overexpression phenotype. Foxo, the forkhead transcriptional factor involved in the c-Jun N-terminal Kinase (JNK) stress-induced pathway, was placed in a UAS-Foxo system with a UAS-CLN3 system to overexpress both proteins in the developing eye of *Drosophila* (Fig. 2C). An overexpression of CLN3 only and an overexpression of foxo only were also performed (Fig. 2A and Fig. 2B). Overexpression of both proteins demonstrated a severely enhanced phenotype, more pronounced than foxo or CLN3 alone. Tuxworth et al. also found that a dominant CLN3 phenotype can be partially suppressed by a heterozygote dose of the foxo allele (Fig 2D). Previously identified protein of interest *falafel* further supports the hypothesis that CLN3 is involved in stress response signaling by suppressing the CLN3 phenotype, when both proteins are expressed together (Fig. 2E). Examining a different stress-response pathway, Tuxworth et al. demonstrated that a heterozygous *keap1* locus intensely suppresses the CLN3 overexpression phenotype (Fig. 2F). Tuxworth et al. also demonstrated that oxidative stress (in the form of reactive oxygen species scavengers Cu2+Zn2+ superoxide dismutase or catalase) with CLN3 overexpression resulted in almost complete suppression of the CLN3 overexpression phenotype. Given that oxidative stress has been previously indicated as a factor in several neurodegenerative diseases, Tuxworth et al. aimed to determine whether mutations in the CLN3 gene would inhibit the ability of *Drosophila* to mount a response against oxidative stress specifically.

To test this hypothesis, *cln3* mutant *Drosophila* (*cln3∆MB1* ) were made to have null function in the CLN3 protein. The naive mutant *Drosophila* showed no phenotypic abnormality. Upon exposure to oxidative stress through three different means, the *cln3* mutants showed a hypersensitive response and their lifespan was significantly reduced (Fig 3. A-C). Re-expression of CLN3 rescued the loss of activity (Fig. 3D). Tuxworth et al. also wanted to determine whether CLN3 had a role in other environmental stresses, and not just a role in oxidative stress response. Control and *cln3* mutant flies showed no significant difference when subjected to thermal stress (Fig. 3G), starvation (Fig. 3H) or viability under osmotic stress (data not shown).

Additional experiments were performed to determine if increased CLN3 expression in wild-type *Drosophila* was adequate enough to increase the oxidative stress response. Previous research by Tuxworth et al. suggested that expression of CLN3 in Dilp neurons (of the brain) signals the JNK signaling pathway, which increases oxidative stress response. Dilp2 protein is responsible for the stress-response signaling. In a series of experiments studying the response to paraquat and overall lifespan, Tuxworth et al. discovered that the UAS-CLN3 “control” actually decreased oxidative stress response and lifespan compared to wild type *Drosophila*. The heterozygote Dilp2-gal4 driver extended lifespan, and the Dilp2-gal4/UAS-CLN3 insertion extended lifespan and showed higher resistance to paraquat (Fig. 3E-F).

To see if reactive oxygen species (ROS) accumulation was the cause of *cln3* mutant failure to mount a stress response, a common indicator of hydrogen peroxide activity was used to quantify ROS in control and mutant flies. *cln3* mutant flies showed increased ROS levels compared after exposure to oxidative stress (Fig. 4A). Green fluorescent protein (GFP) was used to determine whether antioxidant gene transcription was failing in *cln3* mutants, resulting in the ROS accumulation. The GFP reporter gene was induced by Nrf2 transcription factor responding correctly to oxidants. GFP is expressed in Malpighian tubules of *Drosophila* (analogous to the human kidney) following oxidative stress. GFP is expressed in both mutant and control *Drosophila* after a treatment with paraquat; *cln3* mutant flies actually express more GFP than the control. Additional studies indicated that, following an oxidative stress induced by DEM or paraquat, the concentration of CLN3 protein does not change (Fig. 4D-E).

Conclusion/Discussion:

The CLN3 gene has strong interactions with several modifiers that belong to important biological pathways and processes. Among the modifiers is a major group that is involved in stress response signaling. Tuxworth et al. have successfully demonstrated that the CLN3 gene protein in *Drosophila* is important in mounting a cellular stress response to oxidative stress specifically. CLN3 cannot be considered a non-specific stress response protein, as it did not amount a response to thermal stress, starvation, or osmotic pressure. Further, it was determined that CLN3 expressed in neurons and Malpighian tubules is sufficient enough to increase resistance to oxidative pressure and prolong the lifespan of treated *Drosophila. Cln3* mutants were hypersensitive to oxidative treatment and suffered from fatality sooner than control *Drosophila.* Re-expression of CLN3 was able to completely rescue the function of the *cln3* mutant.

Failure to mount an oxidative stress response in *cln3* mutants leads to apoptosis of the wing and degeneration of the eye in *Drosophila.* These phenotypes are characteristic of developments seen in neurodegeneration diseases. *Drosophila* have 4 of 10 likely homologs for genes involved in human NCLs. The function of CLN3, one of these homologs, was previously unknown. The research by Tuxworth et al. demonstrates the role of CLN3 in oxidation stress response and proposes that CLN3 is constitutively expressed to increase the response. Also, it is suggested that CLN3 mutations cause accumulation of ROS, which agrees with previous studies stating that increase in endogenous oxidative load is common in NCL models. Therefore, mutations in the CLN3 gene cause a decreased oxidative stress response that likely results in the neurodegeneration symptoms seen in Batten disease.

Future Research:

Tuxworth et al. have discussed a theory that CLN3 is constitutively expressed in the Malpighan tubules, and it would be interesting to see additional data proving the latter. The mechanism of accumulation of ROS in *cln3* mutants was not determined, so it is necessary to examine what processes are involved in this lack of response when CLN3 protein is absent. In the future, I would like to see Tuxworth et al. work with a different model system; perhaps a mouse model, which may show an increased number of ortholog Batten disease genes to the human genome. Conducting similar ROS accumulation studies in mice may offer more concrete results and offer a better insight in the human Batten disease. Also, Tuxworth et al. theorized that CLN3 is constitutively expressed and it would be interesting to see if this is the case in neurons as well as other tissues. As an early onset disease, a treatment for Batten disease should be a high priority, as there is currently no cure for stopping or reversing the effects of this inherited fatal disease. Future research should also include determination of cellular function of CLN3 in the human genome, as it is still unclear and therefore needs further study.

Although the research of Tuxworth et al. was generally thorough, I would have liked to see more information regarding the modifiers of CLN3 expression. Tuxworth et al. identified many suppressors and enhancers of the overexpression CLN3 phenotype; however they only expanded on the stress-signaling pathway elements. It would be interesting to see which pathways are affected by modifiers that are not part of a stress-signaling pathway (perhaps they are part of an apoptosis pathway?). Also, Tuxworth et al. consistently used the UAS-CLN3 model, where suppressors and enhancers modified the phenotype. In these experiments, the suppression of the overexpressed CLN3 phenotype became close to the wild type phenotype. Although this was an interesting approach, I would have liked to see what suppression of the wild type phenotype looked like, as this is the phenotype that leads to neurodegeneration. Finally, I would have liked to see a connection made between the accumulations of ROS in *cln3* mutants after oxidative stress with the initiation of neurodegeneration. Batten disease is characterized by lipofuscin accumulation that leads to neural degradation and Tuxworth et al. did not make this link in their publication.

Citation:

Tuxworth, R.I., Chen, H., Vivancos, V., Carvajal, N., Huang, X., and G. Tear. (2011). The Batten disease gene *CLN3* is required for the response to oxidative stress. *Human Molecular Genetics*, 20(10); 2037-2047.