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Hwanwook Seong March 31, 2022

Towards identifying genetic modifiers of Fragile X-associated tremor/ataxia syndrome

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Abstract

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The fragile X mental retardation 1 (*FMR1*) gene encodes the fragile X mental retardation protein (FMRP), which is vital for neuronal plasticity and the construction of neural networks. Fragile X-associated tremor/ataxia syndrome (FXTAS) is a late-onset neurodegenerative disorder that is characterized by complications in movement and cognition affecting premutation carriers (55-200 CGG repeats) of the *FMR1* gene. Expanded CGG repeat RNA has been shown to play key roles in FXTAS pathogenesis. There is little insight into the modulation of metabolic pathways in relation to FXTAS. Exploring the changes in these mechanisms can pave new avenues for identifying effective biomarkers and understanding the pathogenesis of the disease. Lysosomes are organelles containing digestive enzymes necessary for cellular degradation, cell signaling, and metabolism. Deficient genes encoding lysosomal proteins can lead to lysosomal storage diseases (LSDs), which can lead to the buildup of toxic cellular materials. To identify the role of lysosome enzymes in FXTAS, this study analyzed a list of LSD-associated genes to genetically screen them for modifying the neuronal toxicity associated with CGG repeats in FXTAS models. The screen identified 11 candidate genes to express at least 60% enhancement in modulating CGG repeat toxicity. Of the 11 candidate genes, *PEX12, GALNS, and GNS* identify phytanic acid peroxisomal oxidation degradation and glycosaminoglycan degradation to be significantly dysregulated pathways. The knockdown of *PEX12* resulted in elevated levels of 2-oxoglutarate and pristanate in modulating the phytanic acid peroxisomal oxidation pathway. Knockdown of *GALNS* identified N-acetyl galactosamine to be significantly downregulated in chondroitin sulfate degradation while knockdown of *GNS* identified 6-Deoxy-L-galactose and D-galactose

significantly downregulated in keratan sulfate degradation. These results indicate the potential causal role of *PEX12, GALNS,* and *GNS* in FXTAS pathogenesis uncovering further understanding of how these genes can act as genetic modifiers of FXTAS. This study aims to analyze how various biochemical pathways in the lysosome and peroxisome can provide insight into the genetic relationship of LSD-associated genes with CGG repeat neurotoxicity in FXTAS.

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Tables and Figures

Introduction

Fragile X-associated tremor/ataxia syndrome (FXTAS) is an adult-onset neurodegenerative disorder that is characterized by poorly coordinated movement, tremor, and parkinsonism (Cabal-Herrera et al., 2020). Damage to the cerebellum, the brain region controlling movement, is commonly observed in affected patients. Typically, individuals contain 5-54 CGG repeats in the 5' untranslated region (UTR) of the *FMR1* gene on the X chromosome; however, individuals of the premutation carrier contain 55-200 CGG repeats and those with the full mutation contain over 200 CGG repeats leading to the development of fragile X syndrome (Mila et al., 2018). FXTAS is seen predominantly in males over the age of 50 where 1:450 males contain the genetic change in the *FMR1* gene, and approximately 40% of them develop the condition. In females, 1:200 females contain the genetic change, but an estimated 16% develop the disorder (Kong et al., 2019).The frequency of an individual developing FXTAS displays incomplete penetrance of the disorder, placing an obstacle for research about the molecular factors leading to FXTAS (Kong et al., 2019). Studying FXTAS pathogenesis will aid in identifying effective biomarkers and therapeutic targets to help make predictions about who may develop the disorder spurring great advancement for therapeutic treatment.

Various animal models have been utilized to study the molecular and genetic basis of FXTAS. The Jin Lab utilized a murine model that expressed the premutation form of the CGG repeat expansions in the 5'UTR of the *FMR1* gene (Kong et al., 2019). Additionally, the Jin Lab developed a *Drosophila* FXTAS model to express the CGG repeat to effectively study CGG repeat toxicity. At the molecular level, FXTAS pathogenesis can be characterized by RNA-mediated toxicity as well as repeat-associated non-AUG (RAN) translation. In RNA toxicity, key RNA binding proteins are unable to perform normal physiological functions due to their sequestration to the CGG repeat expansion area (Kong et al., 2017). RAN translation describes

the erroneous ribosomal scanning allowing translation through the CGG repeat, producing a polyglycine protein, FMRpolyG (Mila et al., 2017). These animal models provide pathologic evidence of FXTAS, but my project observes the expression of CGG repeat expansions in *Drosophila* to examine rough eye phenotypes, demonstrating cell death, loss of pigmentation, and ommatidial disruption. Ultimately, this study sets out to investigate the role of LSD-associated genes in FXTAS pathogenesis.

The field of metabolomics has become a rising area of research for studying the pathologic conditions leading to neurodegenerative disorders such as Huntington's, Parkinson's, and Alzheimer's Diseases (Giulivi et al., 2016; Mielke & Lyketsos, 2010; Shao & Le, 2019). Identifying metabolic alterations in the pathogenesis of these disorders can provide valuable insight for studying the genetic, environmental, and physiological elements of the diseases (Shao and Le 2019). Due to the vast roles of essential metabolites in our bodies, analyzing the roles of enzymes and metabolic pathways necessary for proper physiological function can uncover metabolic changes in FXTAS. Due to the short life cycle and efficient screening process, the use of the *Drosophila* model organism simplifies the complex metabolic reprogramming in diseases furnishing experimental findings that could be difficult to achieve from human samples.

Lysosomal storage diseases (LSDs) are a group of metabolic disorders characterized by enzyme deficiencies within the lysosome that result in an accumulation of undegraded substances (Platt et al., 2018; Sun, 2018). Within the broad category of LSDs, disruptions in sphingolipid and purine metabolism provide a novel understanding of how alterations in the mechanistic pathways of sphingolipid metabolism can be related to the modulation of gene expression in neurodegenerative disorders (Grassi et al., 2019; Kong et al., 2019). A recent study by Kong et al. identified eight genetic modifiers of CGG toxicity in neurodegeneration. Major players were *Schlank (Cers5)* and *Sk2 (Sphk1)* by modulating sphingolipid mechanisms;

Ceramide synthase (*CerS*) is a key enzyme that constructs the backbone of all sphingolipids, ceramide, from sphingosine. It was found that *Schlank*, the fly ortholog of *CerS*, modulated the interaction with the CGG repeat expansion observing the enhancement of FXTAS rough-eye phenotypes. *Sk2* is a sphingosine kinase that phosphorylates sphingosine to sphingosine-1 phosphate (S1P). It was found that S1P was necessary for regulating cell growth and suppressing cell death (Olivera & Spiegel, 1993; Zhang et al., 1991). Knockdown of the *Sk2* gene resulted in higher levels of CGG-associated neurotoxicity, due to lower levels of S1P production. (Kong et al., 2019). These findings provide insight into the role of sphingolipids in neurodegeneration. However, there is limited research about LSD-associated genes in relation to FXTAS that could modulate gene expression. This research provides an approach to investigating the role of lysosome enzymes in FXTAS LSD metabolomics via genetic screening using the *Drosophila* model of FXTAS. This study determines the role of LSD-associated genes as genetic modifiers of CGG toxicity in *Drosophila* providing insight into the pathogenesis of other neurodegenerative diseases. Significant changes occur in peroxisomal metabolism and glycosaminoglycan metabolism in the *Drosophila* model. More notably, this study demonstrates that *Pex12* (*PEX12*), *CG18278* (*GNS*), and *CG7408* (*GALNS*), which encode enzymes for peroxisomal protein import, keratan sulfate degradation, and chondroitin sulfate degradation, act as genetic modifiers of CGG toxicity in *Drosophila*.

Materials and methods

LSD-associated gene selection criteria

LSD-associated genes were collected from the Blueprint Genetics Lysosomal Disorders and Mucopolysaccharidosis Panel as well as Invitae. Corresponding fly orthologs and RNAi lines were found for each gene. To aid in filtering the list of genes of interest, genes were selected

based on DIOPT score and Rank (moderate to high). Differential expression in mouse and human models was found as another selection criterion from FXTAS mice RNA-seq (3-month and 6-month-old) data and FXTAS human Neural Progenitor Cell (NPC) data.

Drosophila genetics

Transgenic flies expressing r$(CGG)_{90}$ were characterized by possessing rough eye phenotypes noted by loss of pigmentation, ommatidial destruction, and cell death. All crosses were grown on a standard medium at 25℃. After crosses were completed, progeny was collected and aged to approximately 7 days. A total of 43 lines were crossed, and a genetic screen was utilized by observing eye phenotype, which was visualized via light microscopy and validated by scanning electron microscopy. The gmr-GAL4 and UAS-TRiP fly lines were obtained from the Bloomington Stock Centre (Bloomington, IN, USA).

Identifying FXTAS significant metabolites

After the genetic screening was performed, LSD-associated genes were categorized into groups of 60% enhancement and 40% enhancement. Enzymes, substrates, metabolic pathways, and diseases associated with mutation of LSD-associated genes were identified for those that expressed 40% and 60% enhancement of the CGG toxicity phenotype. The xmsPANDA R Package was utilized to identify metabolite features in various groups (noncarrier men, premutation men displaying FXTAS symptoms, and premutation men without FXTAS symptoms). Pathway enrichment analysis was conducted in Mummichog. After identification of metabolites and their respective pathways in LSD-associated genes, they were cross-referenced with human male FXTAS significant metabolite Mummichog 2.0 annotations for further analysis.

Scanning Electron microscopy

Drosophila were dehydrated with increasing concentrations of ethanol (25%, 50%, 75%, 100%). Flies were then incubated for 1 hour with hexamethyldisilazane (Electron Microscopy Sciences, Hatfield, PA). Flies were dried overnight under a fume hood after removing hexamethyldisilazane. Analysis of fly eyes was conducted using Dual Stage Scanning Electron Microscope DS 130F (Topcon, Tokyo, Japan).

Results

33 LSD-associated genes selected for observing metabolic perturbations in presence of r(CGG)$_{90}$

In order to observe the interplay of LSD-associated genes with CGG toxicity in FXTAS, I compiled a list of genes of interest and their fly orthologs. Each gene was sorted by DIOPT score and rank by using the DIOPT ortholog finder. A total of 85 LSD-associated genes have been identified from the Blueprint Genetic Lysosomal Disorders and Mucopolysaccharidosis Panel and Invitae. Orthologs were filtered as outlined in the Materials and Methods section. Additionally, LSD-associated genes have been cross-referenced with FXTAS RNA-sequencing data in murine models (3-month and 6-month-old) and NPC data in human models to assess differential expression. From the 85 genes, 33 genes were selected to assess the severity of CGG repeat toxicity in the *Drosophila* FXTAS model by knocking down the LSD-associated genes in the FXTAS eye-specific tissue utilizing the UAS-GAL4 system and screening for the enhancement or suppression of eye phenotype.

Table 1. Results of *Drosophila* genetic screen.
FXTAS r(CGG)$_{90}$ flies expressing GAL4 under the eye-specific tissue gmr driver were crossed with LSD-associated RNAi transgenes. Fly progeny expressing the RNAi against the target LSD-associated genes were collected and aged for 7 days. Fly eyes were observed under light microscopy. SEM was utilized for genes of interest (*PEX12*, *GALNS*, and *GNS*). Genetic screening results indicate 26 genes that enhanced the CGG repeat toxicity of the rough eye phenotype in FXTAS *Drosophila* and 7 genes that expressed no change in eye phenotype. 15 genes expressed at least 40% enhancement of the CGG repeat toxicity (blue) and 11 genes expressed at least 60% enhancement CGG repeat toxicity (orange) in FXTAS *Drosophila*.

No.	Gene		Drosophila Ortholog	BDSC Stock	Screen Phenotype
1	*ALDH5A1*	Succinate-semialdehyde dehydrogenase	*Ssadh*	55683	No change
2	*AMT*	Aminomethyltransferase	*CG6415*	51867	Enhanced
3	*ATP13A2*	Polyamine-transporting ATPase 13A2	*ANNE*	30499	Enhanced
4	*CLN3*	Battenin	*CLN3*	35734	Enhanced
5	*CLN8*	Protein CLN8	*cg17841*	34948	Enhanced
6	*CTSD*	Cathepsin D	*cathD*	53882 55178	No change
7	*DPYD*	Dihydropyrimidine dehydrogenase	*su(r)*	53339	No change
8	*GBA*	Lysosomal acid glucosylceramidase	*Gba1a*	38379	Enhanced
9	*GCDH*	Glutaryl-CoA dehydrogenase	*CG9547*	53327	Enhanced
10	*GLDC*	Glycine dehydrogenase	*CG3999*	57487	No change
11	*GNS*	N-acetylglucosamine-6-sulfatase	*CG18278*	28520	Enhanced
				51878	No change
12	*HEXA*	Beta-hexosaminidase subunit alpha	*Hexo1*	67312	No change
			fdl	28298	Enhanced
13	*HRAS*	GTPase HRas	*Ras85D*	34619	Enhanced
14	*LAMA2*	Laminin subunit alpha-2	*Wb*	29559	Enhanced
15	*LAMP2*	Lysosome-associated membrane glycoprotein 2	*Lamp1*	35675 38254 38335 55219	Enhanced
16	*LDB3*	LIM domain-binding protein 3	*ZASP52*	31561	Enhanced
17	*MAN2B1*	Lysosomal alpha-mannosidase	*Lmanll*	53294	Enhanced
18	*MANBA*	Beta-mannosidase	*beta-Man*	53272	Enhanced
19	*MFSD8*	Major facilitator superfamily domain-containing protein 8	*Cln7*	55664	No change
20	*NPC1*	NPC intracellular cholesterol transporter 1	*Npc1a*	37504	Enhanced
21	*NPC2*	NPC intracellular cholesterol transporter 2	*Npc2a*	38237	Enhanced
22	*PEX10*	Peroxisome biogenesis factor 10	*Pex10*	51826	Enhanced

23	*PEX12*	Peroxisome assembly protein 12	*Pex12*	53308	Enhanced
24	*PEX16*	Peroxisomal membrane protein PEX16	*Pex16*	57495	Enhanced
25	*PEX3*	Peroxisomal biogenesis factor 3	*Pex3*	50694	No change
26	*PEX5*	Peroxisomal targeting signal 1 receptor	*Pex5*	55322	Enhanced
27	*PGK1*	Phosphoglycerate kinase 1	*PGK*	33632	Enhanced
28	*PPT1*	Palmitoyl-protein thioesterase 1	*Ppt1*	55331	No change
29	*SUMF1*	Formylglycine-generating enzyme	*CG7049*	51896	Enhanced
30	*TCF4*	Transcription factor 4	*Da*	26319 35686	Enhanced
31	*DNAJC5*	DnaJ homolog subfamily C member 5	*Csp*	33645	Enhanced
32	*PEX1*	Peroxisome biogenesis factor 1	*Pex1*	28979	Enhanced
33	*GALNS*	N-acetylgalactosamine-6-sulfatase	*CG18278* *CG7408* *CG30059*	28520 51878 65359 28607	Enhanced

Table 2. 11 candidate genes that exhibit 60% enhancement of r(CGG)$_{90}$ in *Drosophila*

No.	Gene		Drosophila Ortholog	BDSC stock	Effect on r(CGG)90 toxicity
1	*ATP13A2*	Polyamine-transporting ATPase 13A2	*Anne*	30499	Enhanced
2	*CLN3*	Battenin	*Cln3*	35734	Enhanced
3	*GNS*	N-acetylglucosamine-6-sulfatase	*CG18278*	28520	Enhanced
4	*MANBA*	Beta-mannosidase	*beta-Man*	53272	Enhanced
5	*NPC1*	NPC intracellular cholesterol transporter 1	*Npc1a*	37504	Enhanced
6	*PEX16*	Peroxisomal membrane protein PEX16	*Pex16*	57495	Enhanced
7	*PGK1*	Phosphoglycerate kinase 1	*PGK*	33632	Enhanced
8	*TCF4*	Transcription factor 4	*da*	35686	Enhanced
9	*DNAJC5*	DnaJ homolog subfamily C member 5	*Csp*	33645	Enhanced
10	*PEX12*	Peroxisome assembly protein 12	*Pex12*	53308	Enhanced
11	*GALNS*	N-acetylgalactosamine-6-sulfatase	*CG7408*	65359	Enhanced

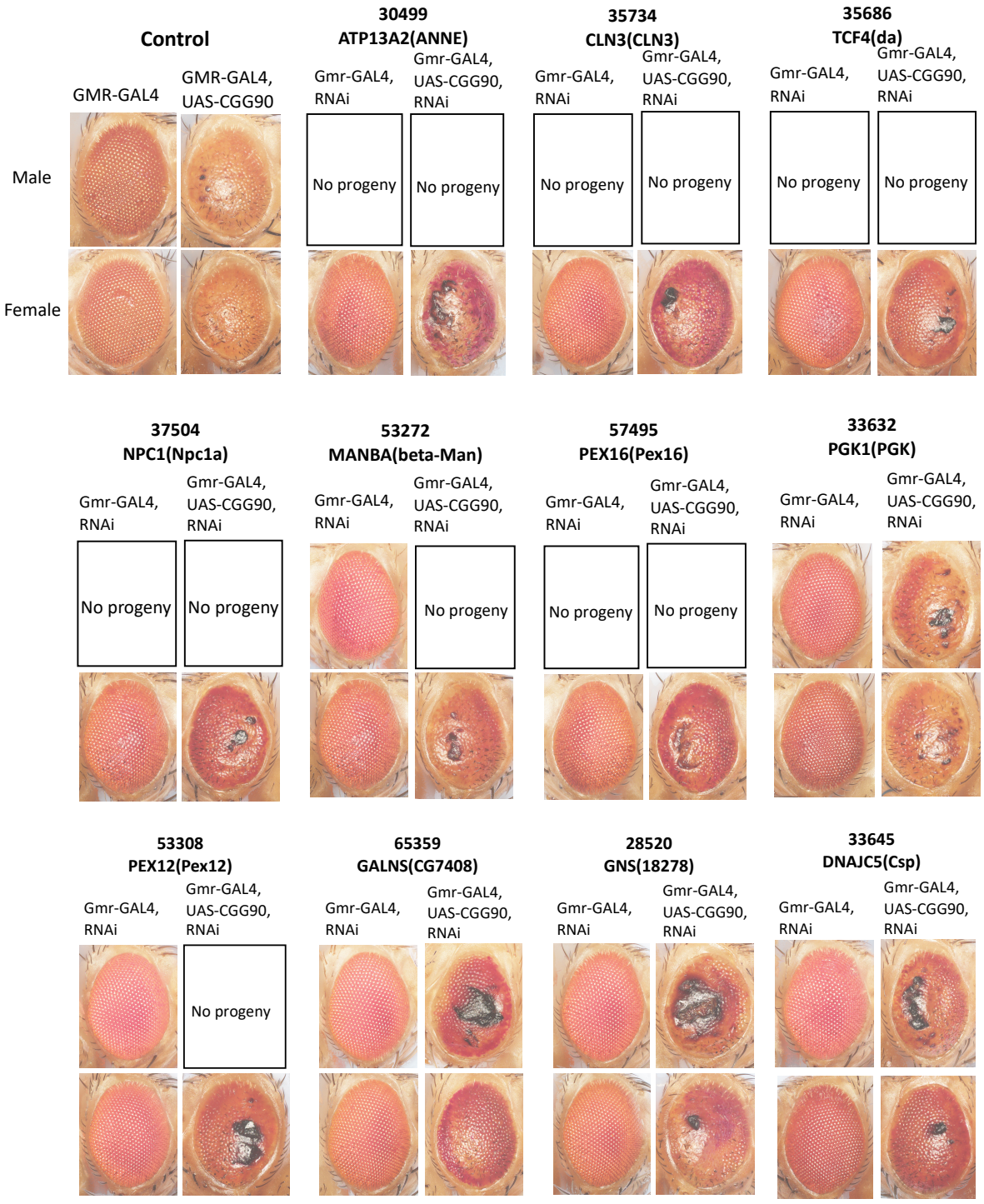

Figure 1. Knockdown of 11 LSD-associated genes result in enhancement of r(CGG)$_{90}$ neurodegeneration observed in FXTAS *Drosophila*. Control panel and each LSD-associated gene categorized by sex (male on first row and female on second row). WT control (column 1 in control panel), r(CGG)$_{90}$ control (column 2 in control panel). For each LSD-associated gene: WT fly expressing LSD-KD gene (gmr-GAL4, RNAi) and (CGG)$_{90}$ fly expressing RNAi knockdown LSD-KD gene (gmr-GAL4 UAS-CGG90, RNAi).

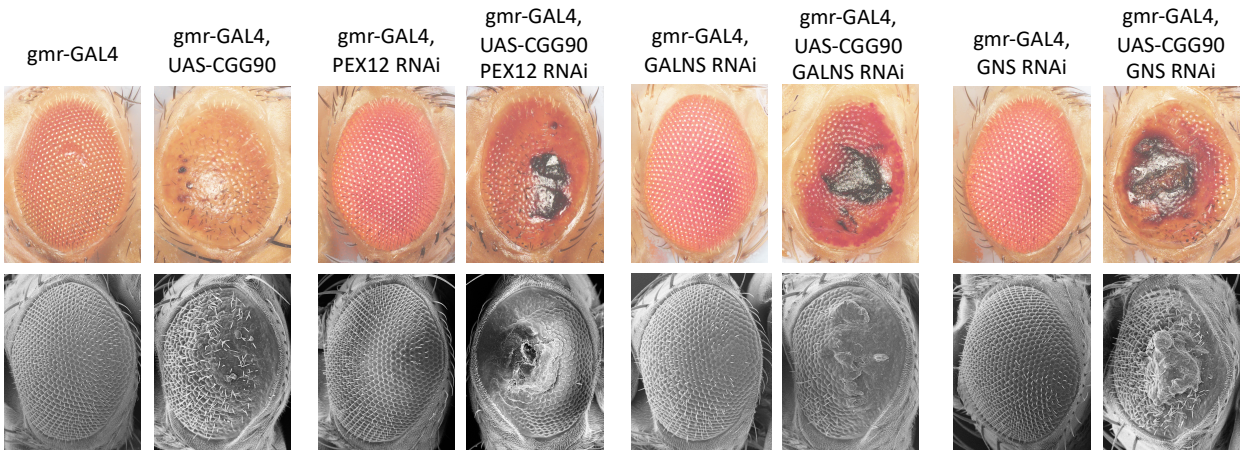

Figure 2. Knockdown of genes of interest (*PEX12, GALNS*, and *GNS*) enhance CGG repeat toxicity-mediated neurodegeneration in FXTAS *Drosophila* models. Top panel displays light microscopy images from *Drosophila* with corresponding genotypes of LSD-associated genes crossed with gmr-GALR4 flies and gmr-GAL4, UAS$(CGG)_{90}$ flies. Bottom panel displays scanning electron microscopy (SEM) images. Controls: WT control (gmr-GAL4) (column 1), $(CGG)_{90}$ control (gmr-GAL4, UAS$(CGG)_{90}$) (column 2). Columns 3 and 4 show RNAi knockdown of *PEX12* in WT and $(CGG)_{90}$ flies, respectively. No change in phenotype was observed in WT flies expressing *PEX12* RNAi but enhancement of CGG repeat toxicity in $(CGG)_{90}$ flies expressing *PEX12* RNAi. Columns 5 and 6 show RNAi knockdown of *GALNS* in WT and $(CGG)_{90}$ flies, respectively. No change in phenotype was observed in WT flies expressing *GALNS* RNAi but enhancement of CGG repeat toxicity in $(CGG)_{90}$ flies expressing *GALNS* RNAi. Columns 7 and 8 show RNAi knockdown of *GNS* in WT and $(CGG)_{90}$ flies, respectively, demonstrating no change in phenotype in WT flies expressing *GNS* RNAi but enhancement of CGG repeat toxicity in $(CGG)_{90}$ flies expressing *GNS* RNAi.

Drosophila genetic screening identifies 11 candidate genes that exhibit significant enhancement with FXTAS CGG repeat toxicity

Out of the 33 selected LSD-associated genes, the *Drosophila* screen identified 11 candidate genes that exhibited at least 60% enhancement of the CGG toxicity in FXTAS *Drosophila* (Table 1). Knockdown of all 11 genes resulted in enhancement of the rough eye phenotype (Table 2). *Anne, Cln3, CG18278, beta-Man, Npc1a, Pex16, PGK, da, Csp, Pex12, and CG7408* are *Drosophila* orthologs of Polyamine-transporting ATPase 13A2 (*ATP13A2*), Battenin (*CLN3*), N-acetylglucosamine-6-sulfatase (*GNS*), Beta-mannosidase (*MANBA*), NPC intracellular

cholesterol transporter 1 (*NPC1*), Peroxisomal membrane protein PEX16 (*PEX16*), Phosphoglycerate kinase 1 (*PGK1*), Transcription factor 4 (*TCF4*), DnaJ homolog subfamily C member 5 (*DNAJC5*), Peroxisome assembly protein 12 (*PEX12*), and N-acetylgalactosamine-6-sulfatase (*GALNS*), respectively (Figure 1). In summary, 11 genes have been marked for significantly modulating CGG toxicity in FXTAS *Drosophila* by exhibiting at least 60% enhancement (Table 2). However, 15 genes have also been identified to exhibit 40% enhancement of the CGG toxicity eye phenotype (Table 1). For those LSD-associated genes with at least 60% enhancement, this study centralizes on the *PEX12*, *GALNS*, and *GNS* genes to study their involvement in metabolic pathways and how they modulate CGG repeat toxicity.

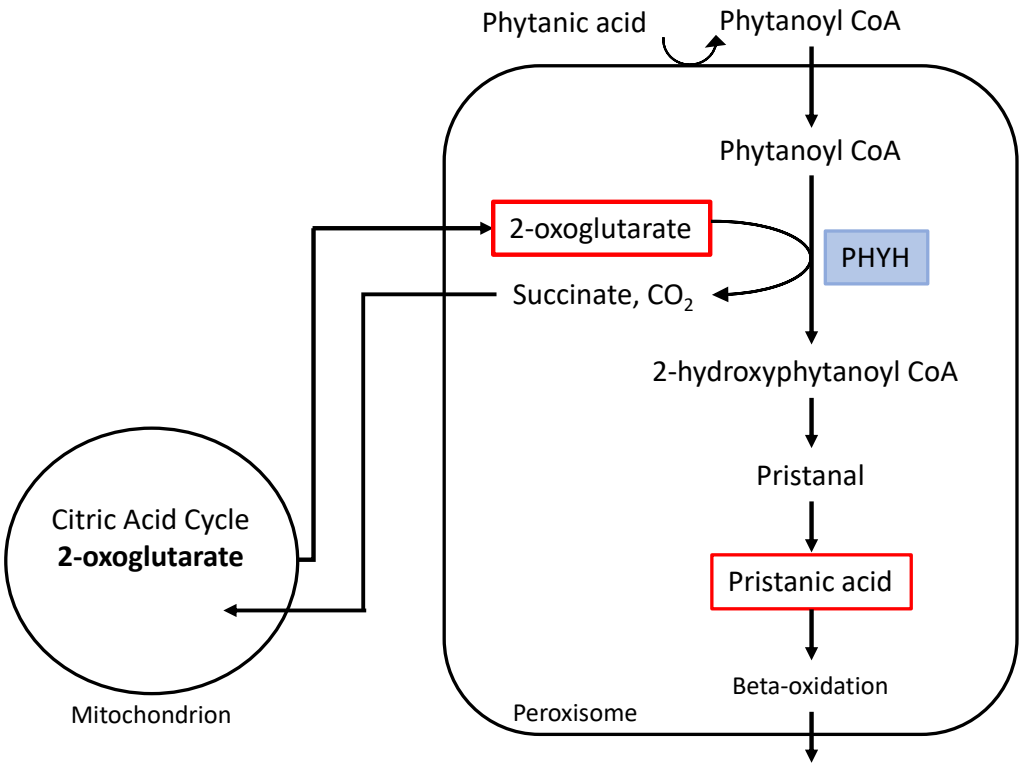

Figure 3. Phytanic acid peroxisomal oxidation pathway significantly altered in FXTAS. Significant metabolites from FXTAS untargeted metabolic profiling highlighted in red rectangles. 2-Oxoglutarate ($p = 0.0121$) and pristanate ($p = 0.0091$) were significantly elevated in FXTAS.

Branched-Chain Fatty Acid Catabolism may be perturbed in FXTAS: knockdown of *Pex12* (PEX12) results in enhancement of CGG repeat toxicity

To discern genetic interactions of the LSD-KD genes, genetic screening was employed with the *Drosophila* model of FXTAS. Using the efficient UAS-GAL4 system, knockdown of *PEX12* (*Pex12*) resulted in a 65% enhancement of the CGG repeat toxicity phenotype (Figure 2). The PEX genes are a network of genes that are essential for the function of peroxisomes. Various biochemical pathways have been identified in peroxisomal function such as very-long-chain fatty acid metabolism, pipecolic acid metabolism, phytanic acid metabolism, plasmalogen biosynthesis, and glyoxylate detoxification (Wangler et al., 2018). Moreover, Hardin noted phytanic acid peroxisomal oxidation has been identified to be a statistically significant ($p = 0.009$) metabolic pathway seen in FXTAS premutation men vs. noncarrier men comparisons (Hardin et al. 2021). The phytanic acid peroxisomal oxidation metabolic pathway was statistically significant in this study, remaining consistent with Hardin's findings (Figure 4). Wangler et al. (2018) have been able to show that individuals with a mutation in *PEX12* contained elevated levels of phytanic acid and pristanic acid (Figure 3). An accumulation of these branched-chain fatty acids has toxic effects and plays important roles in disorders like Refsum Disease, Peroxisomal Biogenesis Disorder, and Zellweger Syndrome. Specifically, a mutation in *PEX12* results in a disorder known as Peroxisomal Biogenesis Disorder 3B with individuals displaying symptoms like ataxia, psychomotor retardation, hypotonia, etc.

The Jin Lab developed untargeted metabolic profiling data from a cohort of 38 human males with FXTAS diagnosis (cases), 41 males containing the premutation with no FXTAS diagnosis (control), and 20 males characterized as non-carriers (no premutation). Metabolites were identified from blood plasma in the FXTAS case vs. control cohort by utilizing the XMS PANDA R Package for metabolite differential abundance analysis. Two statistically significant

metabolites have been sourced from FXTAS untargeted metabolic profiling data in phytanic acid metabolism. Elevated levels of pristanate ($p = 0.0091$) were identified in male FXTAS cases vs. male non-carrier groups and elevated levels of 2-oxoglutarate ($p = 0.0121$) were observed in male FXTAS cases vs. control comparisons. Pristanate is a deprotonated form of pristanic acid; 2-oxoglutarate originates from the mitochondria through the citric acid cycle and gets integrated as a substrate for a reaction upstream of phytanic acid metabolism. Data collected from FXTAS metabolic profiling correlates to findings from Wangler et al. (2018), which provides insight that pristanate and 2-oxoglutarate are involved in phytanic acid metabolism to potentially modulate CGG repeat toxicity.

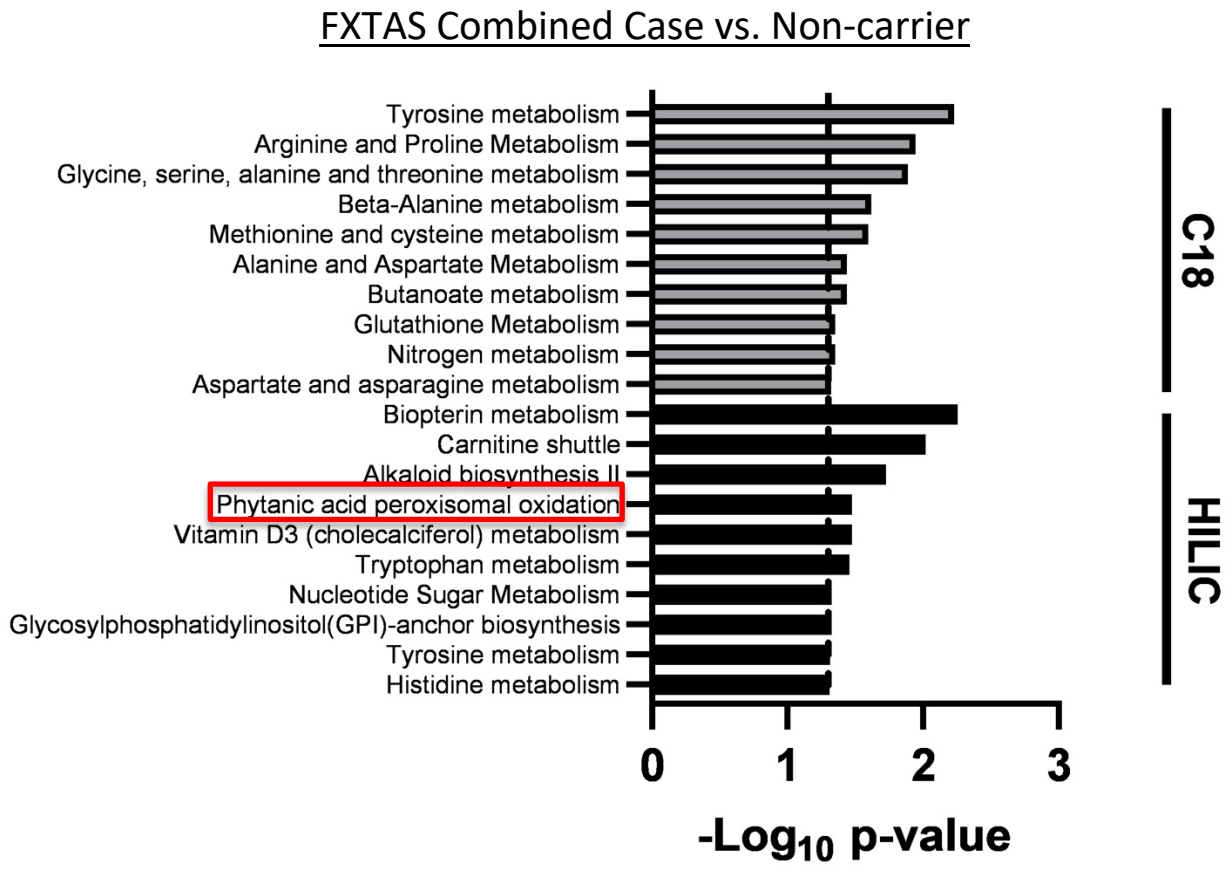

Figure 4. Significantly altered metabolic pathways in human male FXTAS combined case vs. non-carrier comparisons. Phytanic acid peroxisomal oxidation identified to be a significantly altered pathway in FXTAS (highlighted in red box). C18 column of HPLC

represents pathways separating more hydrophobic compound. HILIC column of HPLC represents pathways separating more hydrophilic compounds. Pathway enrichment (Metabolite Set Enrichment Analysis) created using Mummichog 2.0. Adjusted p-values transformed with -log10. Cutoff for significance is $p < 0.05$. Dashed line marks -log10 p-value of 1.301.

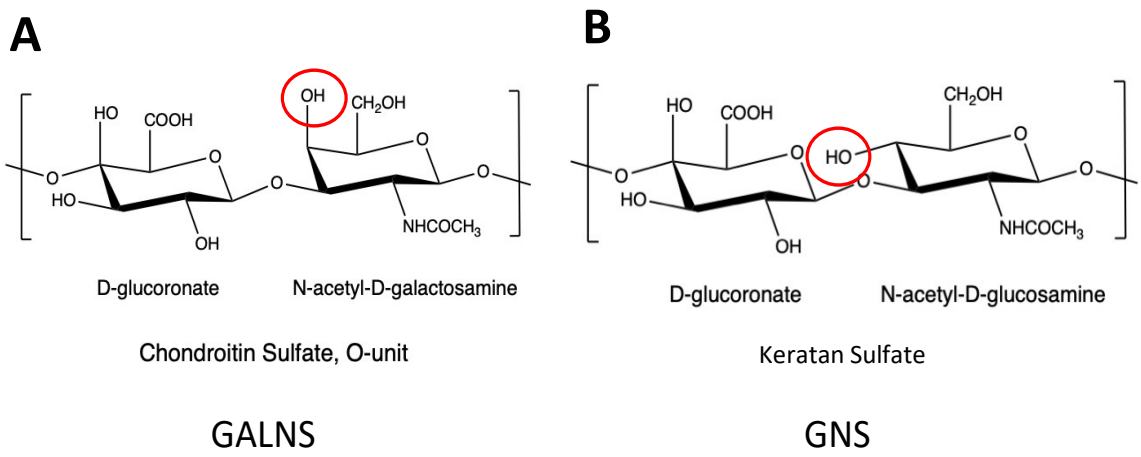

Figure 5. Glycosaminoglycan structures in chondroitin sulfate and keratan sulfate. (A) Hydroxy group in axial position in N-acetyl-D-galactosamine unit of chondroitin sulfate (red circle) (B) Hydroxy group in equatorial position in N-acetyl-D-glucosamine unit of keratan sulfate.

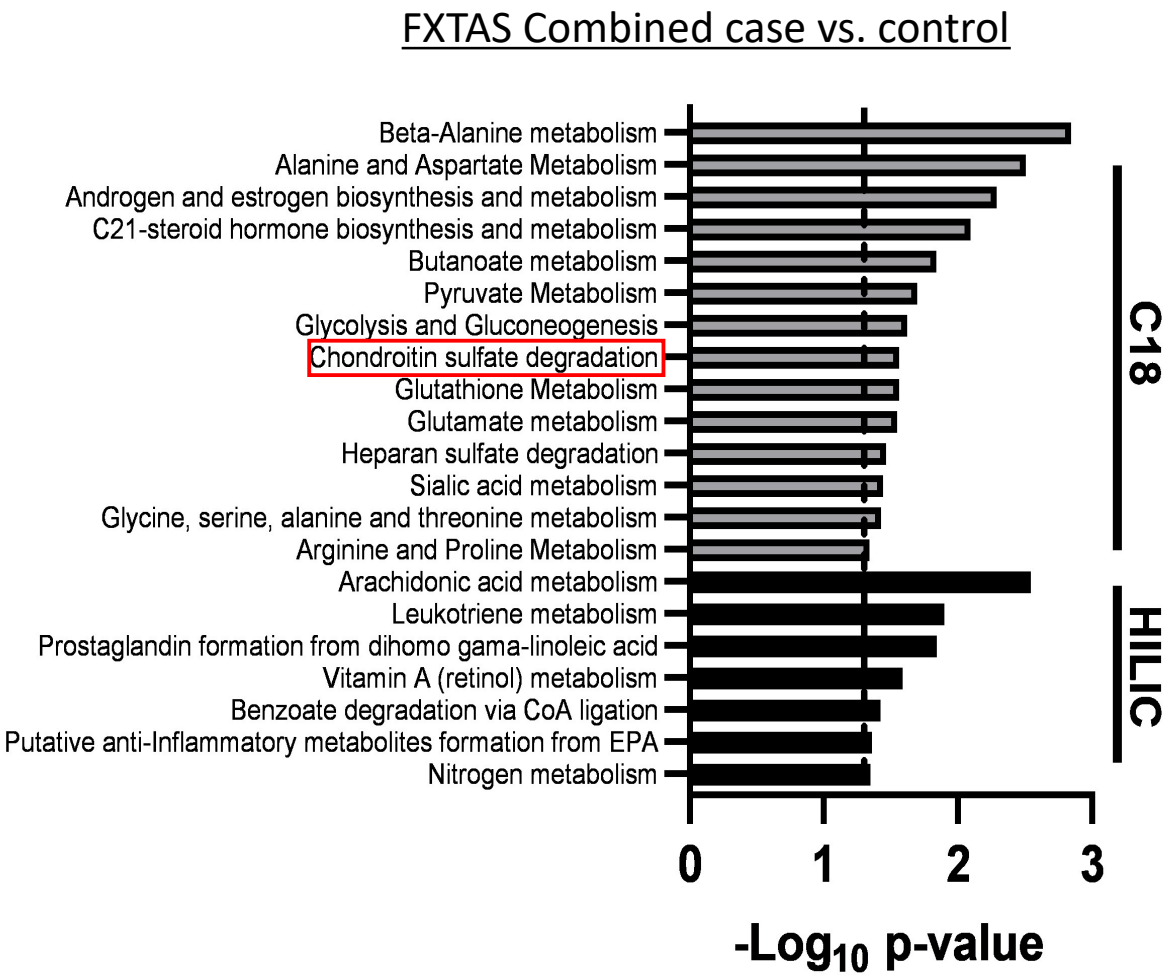

Figure 6. Significantly altered metabolic pathways in human male FXTAS combined case vs. control comparisons. Chondroitin Sulfate degradation identified to be a significantly altered pathway in FXTAS (highlighted in red box). C18 column of HPLC represents pathways separating more hydrophobic compound. HILIC column of HPLC represents pathways separating more hydrophilic compounds. Pathway enrichment (Metabolite Set Enrichment Analysis) created using Mummichog 2.0. Adjusted p-values transformed with -log10. Cutoff for significance is $p < 0.05$. Dashed line marks -log10 p-value of 1.301.

Glycosaminoglycan (GAG) degradation pathways may be involved in FXTAS pathogenesis: knockdown of *GALNS* and *GNS* result in enhancement of the CGG toxicity

In addition to phytanic acid metabolism, metabolites in glycosaminoglycan (GAG) degradation were found to be significantly altered in the presence of CGG repeats. GAGs include a variety of sugars such as chondroitin sulfate, keratan sulfate, dermatan sulfate, and heparan sulfate. In particular, *GALNS* and *GNS* genes are actively involved in chondroitin sulfate degradation and keratan sulfate degradation, respectively. *GALNS* is responsible for producing an enzyme known as N-acetylgalactosamine-6-sulfatase, which removes the sulfate group on the sixth carbon position in the N-acetyl galactosamine unit of chondroitin sulfate (Figure 5A). *GNS* is responsible for producing an enzyme known as N-acetylglucosamine-6-sulfatase, which removes the sulfate group on the sixth carbon position in the N-acetylglucosamine unit of keratan sulfate (Figure 5B). GAG degradation pathways have been identified to be significantly altered in FXTAS case vs. control comparisons, specifically chondroitin sulfate degradation (Figure 6); therefore, investigating the mechanics of this metabolic pathway can aid in understanding how *GALNS* and *GNS* are involved with modulating CGG repeat toxicity.

Both genes have been identified to significantly enhance the CGG phenotype in *Drosophila* eyes through genetic screening (GALNS = 75% enhancement, GNS = 84.21% enhancement, Figure 2). Utilizing the FXTAS untargeted metabolic profiling data, a significant ($p = 0.0254$) downregulation of the metabolite N-acetyl galactosamine was observed in FXTAS case vs. control comparisons. N-acetyl galactosamine is part of the disaccharide unit in chondroitin sulfate and serves as a substrate for sulfation on the sixth carbon position. Additionally, FXTAS metabolic profiling of case vs. control comparisons has identified 6-Deoxy-L-galactose ($p = 0.0360$) and D-galactose (0.0276) metabolites to be significantly downregulated in keratan sulfate degradation. D-galactose is part of the repeating disaccharide

unit in keratan sulfate. Although N-acetylglucosamine-6-sulfatase does not act directly on D-galactose, D-galactose is an essential component of keratan sulfate (Figure 5B). The decrease in N-acetyl galactosamine coinciding with the decrease in D-galactose in FXTAS case vs. control led to the hypothesis that *GALNS* and *GNS* function may be significantly altered in FXTAS.

Discussion

Prior studies have noted the importance of utilizing metabolomics to reveal significant perturbations in metabolic pathways in the pathogenesis of diseases like FXTAS. Modulating pathways in phytanic acid peroxisomal oxidation, chondroitin sulfate degradation, and keratan sulfate degradation served to be novel metabolic pathways to intuit the potential causal relationship of their interaction with CGG repeat toxicity.

In this study, it has been demonstrated that *Drosophila* genetic screening has been successful in identifying genetic modifiers alongside the support of metabolic profiling data to functionally analyze the genes' roles in interaction of CGG repeats. The results of this study indicated 11 candidate genes to significantly modulate CGG repeat toxicity with at least 60% enhancement, specifically interpreting *PEX12*, *GALNS*, and *GNS* (Figure 1, Figure 2). Up to now, there has been little discussion about the systematic understanding of how these genes contribute to FXTAS pathogenesis.

Other biochemical pathways have been significantly associated with modulating CGG repeat toxicity in FXTAS such as sphingolipid metabolism and purine metabolism. The current study provides alternative perspectives in FXTAS pathogenesis. Phytanic acid peroxisomal degradation is one of the various peroxisomal biochemical pathways. In accordance with the present results, a previous report has demonstrated that phytanic acid peroxisomal degradation was a statistically significant dysregulated pathway in FXTAS premutation men vs. noncarrier

men (Hardin et al., 2021, Figure 4). *Drosophila* genetic screening reflected strong interaction of *PEX12* and the CGG repeats, displaying a significant effect on the fly eye (Figure 2). It has been stated that individuals with a mutation in *PEX12* are diagnosed with Peroxisomal Biogenesis Disorder 3B (PBD 3B), displaying symptoms like ataxia and psychomotor retardation, which relate to phenotypes seen in FXTAS individuals (Wangler et al, 2018). Furthermore, they have sourced those patients with PBD 3B contained elevated levels of phytanic acid and pristanic acid. Some of these results correspond to findings in this study, particularly focusing on 2-oxoglutarate ($p = 0.0121$) and pristanate ($p = 0.0091$) to be significantly altered metabolites in phytanic acid peroxisomal oxidation (Figure 3). Untargeted metabolic profiling of FXTAS case vs. control comparisons identified these metabolites to be statistically significantly altered. 2-oxoglutarate is synthesized in the citric acid cycle in the mitochondria that gets transported to the peroxisome to partake in phytanic acid metabolism. The phytanoyl-CoA hydroxylase enzyme is critical in converting phytanoyl-CoA to 2-hydroxyphytanoyl-CoA by utilizing 2-oxoglutarate as a substrate in phytanic acid degradation. On another note, pristanic acid is a downstream product of phytanic acid degradation that gets integrated in peroxisomal beta-oxidation for further metabolism. FXTAS metabolic profiling additionally identified pristanate to be elevated in phytanic acid metabolism, which correlates to findings of individuals with a mutation in *PEX12* (Wangler et al., 2018). Altogether, genetic screening and metabolic profiling collectively support the hypothesis that phytanic acid metabolism is involved with FXTAS pathogenesis, reporting elevated levels of 2-oxoglutarate and pristanate metabolites in association with neurotoxicity.

Another alternative biochemical pathway explored in this study revealed that glycosaminoglycan (GAG) degradation may be involved in perturbing FXTAS. In particular, *GALNS* and *GNS* have been identified to be actively involved in chondroitin sulfate and keratan sulfate degradation, which are types of GAGs (Chin et al., 2020; Tomatsu et al., 2004, 2005).

GALNS is responsible for producing an enzyme known as N-acetyl galactosamine-6-sulfatase, which removes the sulfate group on the sixth carbon position of N-acetyl galactosamine. *GNS* is responsible for producing an enzyme known as N-acetyl glucosamine-6-sulfatase, which removes the sulfate group on the sixth carbon position of N-acetyl glucosamine. When the *GALNS* gene does not function properly, this results in a disorder known as Mucopolysaccharidosis type IVA (MPS IVA). Borlot et al. (2014) has identified that within their cohort of patients, a common symptom was motor weakness, which correlates to symptoms displayed with FXTAS patients. Mutations in *GNS* results in a disorder called Mucopolysaccharidosis type IIID (MPS IIID). Similarly, individuals with this disorder display motor developmental issues. Among the statistically significant altered pathways in FXTAS case vs. control comparisons, chondroitin sulfate degradation was identified (Figure 6). Utilizing FXTAS metabolic profiling data, a significant ($p = 0.0254$) downregulation of the metabolite N-acetyl galactosamine (GalNAc) was observed. GalNAc is part of the repeating disaccharide unit in chondroitin sulfate, which is essential for the makeup of the GAG. Since this metabolite is significantly downregulated in FXTAS, it can be deduced that the knockdown of *GALNS* will further reduce the ability for its enzyme to process the metabolite, supporting the hypothesis that *GALNS* deficiency contributes to the enhancement of CGG toxicity. Regarding *GNS*, significant metabolites like 6-Deoxy-L-galactose ($p = 0.0360$) and D-galactose ($p = 0.0276$) have been located by untargeted metabolic profiling data. Their involvement with chondroitin sulfate degradation remains to be in discussion. However, D-galactose serves to be part of the repeating disaccharide unit in keratan sulfate. Keratan sulfate degradation has not been identified as a statistically significant altered pathway in FXTAS case vs. control, but 6-Deoxy-L-galactose and D-galactose can be analyzed in future analyses as to how they are involved with interacting with CGG repeats.

This study set out with the aim of assessing how LSD-associated genes can be identified as potential genetic modifiers of CGG toxicity in FXTAS pathogenesis. Up to date, no biomarkers of FXTAS have been identified, hindering therapeutic strategies and prognosis of FXTAS patients. The results of this study identified 3 LSD-associated genes and 5 metabolites to be significantly altered promoting further research in the exploration of biomarkers for FXTAS.

In this study, untargeted metabolic profiling alongside facile *Drosophila* genetic screening functionally validated interaction of LSD-associated genes with CGG repeat toxicity. The broad field of metabolomics and its complex nature may pose as obstacles for furthering progress of FXTAS research; however, this study explores the strength of using efficient, inexpensive, and high-throughput analysis of *Drosophila* genetic screening to validate metabolic information gathered from untargeted metabolic profiling. Several questions remain unanswered. *PEX12* may be a potential biomarker for further analysis, but how does the network of PEX genes (*PEX16, PEX10, PEX5*) interact with r(CGG)$_{90}$ to modulate FXTAS pathogenesis (Zeharia et al., 2007)? Regarding *GALNS*, how does an overexpression of this gene differ from this study's results of downregulation to modulate CGG toxicity? The deficiency in the *GNS* gene has also been associated with other metabolic pathways such as Butanoate metabolism and Arginine and Proline Metabolism (Tebani et al., 2018). How do these metabolic pathways differ from the insights gathered from keratan sulfate degradation observed in this study? Further work is required to establish the viability of the results acquired in this study, but the applicability of *Drosophila* genetic screening can be assessed in various human diseases.

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