Heparan Sulfate Structure Affects Autophagy, Lifespan, Responses to Oxidative Stress, and Cell Degeneration in Drosophila parkin Mutants

Claire Reynolds-Peterson,* Jie Xu,* Na Zhao,* Casey Cruse,* Brandon Yonel,* Claire Trasorras,* Hidenao Toyoda,† Akiko Kinoshita-Toyoda,† Jennifer Dobson,* Nicholas Schultheis,* Mei Jiang,* and Scott Selleck*,†
*Department of Biochemistry & Molecular Biology, The Pennsylvania State University, University Park, PA, 16802 and †Faculty of Pharmaceutical Sciences, Ritsumeikan University, 1-1-1 Nojihigashi, Kusatsu, Shiga, 525-8577, JAPAN
ORCID ID: 0000-0001-6036-8542 (S.S.)

ABSTRACT  Autophagy is a catabolic process that provides cells with energy and molecular building blocks during nutritional stress. Autophagy also removes misfolded proteins and damaged organelles, a critical mechanism for cellular repair. Earlier work demonstrated that heparan sulfate proteoglycans, an abundant class of carbohydrate-modified proteins found on cell surfaces and in the extracellular matrix, suppress basal levels of autophagy in several cell types during development in Drosophila melanogaster. In studies reported here, we examined the capacity of heparan sulfate synthesis to influence events affected by autophagy, including lifespan, resistance to reactive oxygen species (ROS) stress, and accumulation of ubiquitin-modified proteins in the brain. Compromising heparan sulfate synthesis increased autophagy-dependent processes, evident by extended lifespan, increased resistance to ROS, and reduced accumulation of ubiquitin-modified proteins in the brains of ROS exposed adults. The capacity of altering heparan sulfate biosynthesis to protect cells from injury was also evaluated in two different models of neurodegeneration, overexpression of Presenilin and parkin mutants. Presenilin overexpression in the retina produces cell loss, and compromising heparan sulfate biosynthesis rescued retinal patterning and size abnormalities in these animals. parkin is the fly homolog of human PARK2, one of the genes responsible for juvenile onset Parkinson’s Disease. Parkin is involved in mitochondrial surveillance and compromising parkin function results in degeneration of both flight muscle and dopaminergic neurons in Drosophila. Altering heparan sulfate biosynthesis suppressed flight muscle degeneration and mitochondrial dysmorphology, indicating that activation of autophagy-mediated removal of mitochondria (mitophagy) is potentiated in these animals. These findings provide in vivo evidence that altering the levels of heparan sulfate synthesis activates autophagy and can provide protection from a variety of cellular stressors.

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*Corresponding author: 206D Life Sciences Building, University Park PA, 16802. E-mail: sbs24@psu.edu

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Heparan sulfate modified proteins are abundant proteins of the cell surface and extracellular matrix, named for the unbranched and highly sulfated disaccharide polymers covalently attached to the protein core (Bishop et al. 2007; Esko and Selleck 2002; Lindahl et al. 2017). Biosynthesis of heparan sulfate occurs in the golgi and mutations affecting this enzyme machinery compromise the modification of numerous proteins, including glypicans and syndecans, two integral membrane proteoglycans involved in signaling. Studies of genes encoding proteins required for heparan sulfate polymer synthesis and sulfation have been instrumental in defining the activities of heparan sulfate modified proteins. These genes are highly conserved across species, including C. elegans, Drosophila, the mouse, and humans. Heparan sulfate modified
proteins are functionally diverse molecules, regulating growth factor signaling, endocytosis, and the distribution of molecules in the matrix. Both the protein core and the heparan sulfate chains govern these functions. The abundance and broad expression of heparan sulfate modified proteins, together with their diverse functions in modulating signaling, provide the capacity to effect cellular physiology in a myriad of ways. Here we explore their function in regulating autophagy.

Autophagy is important for proteostasis, organelle turnover, and protecting cells from a variety of cellular stresses. In vertebrates basal autophagy is critical for normal cellular health, highlighted by the extensive neuronal death in the cerebrum and cerebellum of mice lacking Atg7, a critical autophagy component (Komatsu et al. 2006). Upregulation of constitutive autophagy increases lifespan in C. elegans (Kapahi et al. 2017) and Drosophila (Simonsen et al. 2008) and can rescue neurons from protein-aggregate toxicity in a number of models, including Drosophila (Wang et al. 2009; Kim et al. 2017). Recent work has demonstrated that increases in basal autophagy regulated by Beclin can also increase lifespan and health span in the mouse (Fernández et al. 2018). There is also evidence that mitophagy, a component of autophagy, is important for removing damaged mitochondria and failure of mitochodrial surveillance has a significant role in the pathology of Parkinson’s disease (de Vries and Przedborski 2013).

In the course of examining heparan sulfate modified protein function at the Drosophila neuromuscular junction we discovered that heparan sulfate synthesis in muscle had profound effects on autophagy. Decreasing heparan sulfate synthesis produced reductions in the number of mitochondria and changes in the structure of post-synaptic specializations, effects shown to be mediated by an increase in autophagy (Reynolds-Peterson et al. 2017). The capacity of heparan sulfate modified proteins to suppress autophagy was also documented in fat body, a critical metabolic sensing and energy storage tissue in Drosophila. There are a number of findings in the mouse that suggest heparan sulfate modified protein-mediated inhibition of autophagy occurs in vertebrates as well. When heparan sulfate accumulates, such as in many lysosomal storage diseases, autophagy is suppressed (Fiorenza et al. 2018; Cox and Cachon-Gonzalez 2012; Bartolomeo et al. 2017; Settembre et al. 2008a). Transgene-mediated expression of a heparan sulfate-degrading enzymes, heparanase (Hpa1), increases autophagy, consistent with an inhibitory role of heparan sulfate modified proteins on autophagy (Ilan et al. 2015; Shteingauz et al. 2015). Conversely, gene knockout of Hpa1 results in suppression of autophagy in multiple tissues, consistent with an inhibitory role of heparan sulfate modified proteins on autophagy levels.

However, specific heparan sulfate-modified proteins have been shown to affect autophagy in distinct manners. Loss of Perlecan increases autophagy in mouse muscle, consistent with an autophagy-inhibitory activity (Ning et al. 2015). However, Endorepellin, a C-terminal fragment of Perlecan, and Decorin, a small leucine-rich proteoglycan, induce autophagy (Gubbiotti and Iozzo 2015; Poluzzi et al. 2014). These studies demonstrate that individual proteoglycans can either inhibit or stimulate autophagy in different cellular contexts. We were interested in the effects of heparan sulfate modified proteins generally on autophagy regulation, and known physiological functions of autophagy, including responses to stress, lifespan, proteostasis, and mitophagy in an intact animal system.

In the studies reported here we examined the influence of heparan sulfate biosynthesis levels and sulfation state on known physiological functions of autophagy. We show that decreasing heparan sulfate levels or sulfation has all the hallmarks of global activation of basal autophagy, increasing resistance to oxidative stress and extending lifespan. We have also examined the capacity of altered heparan sulfate biosynthesis to provide protection from cell loss in a Presenilin model of Alzheimer’s Disease (AD), or defects in mitochondrial surveillance mediated by mutations in parkin, the homolog of PARK2. In both of these models of human neurodegenerative disorders, altering heparan sulfate biosynthesis rescued cell loss, showing that changes in heparan sulfate can affect the capacity of cells to tolerate a variety of cellular stresses.

**METHODS AND MATERIALS**

**Fly rearing and strains**

Fly strains were raised on standard cornmeal/sucrose/agar media at 25°C. Oregon-R, UAS-w-RNAi (30033), and VDRC60100 strains served as controls; stock numbers are listed in parentheses. Unless otherwise specified, when UAS-sf-RNAi is shown, UAS-sf-RNAi HMS00543 (34601) strain was used. RNAi strains and a control strain with the same genetic background were obtained from the Vienna Drosophila RNAi Center (VDRC): UAS-Atg8a-RNAi (43097), UAS-sf-RNAi (5070), UAS-ttv-RNAi (4871), UAS-w-RNAi (30033) and empty vector control (60100). UAS-Atg5-RNAi is Bloomington Stock number 27551.

**UAS-mcherry-Atg8a (37750), elav-Gal4 > UAS-Dcr2 (25750), UAS-Atg8a (10107), UAS-Psr (8309) and the Drosophila Transgenic RNAi Project (TRIP) lines UAS-sf-RNAi GLC01656 (50538), UAS-sf-RNAi HMS00543 (34601), and UAS-mCherry-RNAi (35785) were obtained from the Bloomington Drosophila Stock Center (BDSC).**

**The sf (3844) and tt (tt00681) P-element insertion alleles were generated by the Berkeley Drosophila gene disruption project and have been previously described. The sf (3844) ethylmethanesulfonate-induced point mutation was kindly provided by Norbert Perrimon (Lin and Perrimon 1999), parkin alleles (park1, park21) were obtained from the Bloomington Stock Center. sf (park21) recombinant chromosomes were generated and the presence of sf and parkin alleles determined by back crossing to lethal sf and parkin alleles, as well as by PCR analysis of recombinant animals and QPCR to measure mRNA levels (see Supplemental Figure 2). Four recombinants of the two different sf alleles were generated and evaluated. bot (21) is a point mutation and loss-of-function allele (Takeda et al. 2004). UAS-mitoGFP (P-element insertion on the second chromosome, Bloomington stock number 8442) was used to selectively tag mitochondria and muscle specific expression was achieved using mef2-Gal4 (y[w+] w[+] ); P[w+;+mC]=GALA-Mef2.R;3 on the third chromosome, Bloomington stock number 27390.**

**Lifespan and log-rank analysis**

A typical experiment began with over 100 adult flies, 25-30 per vial, examined for viability on standard media at 25°C, and transferred to new media every 48 hr. Significant deviation in survival curves was calculated first on the pooled genotypes by logrank test using the Mantel-Haenszel method, followed by pair-wise comparison between each experimental genotype and the control using the Gehan-Breslow-Wilcoxon test. No individuals were censored during the survival assay, and survival curves of the experimental genotypes do not cross that of the control.

**Oxidant exposure**

Hydrogen peroxide toxicity was performed to observe ROS sensitivity. One-week old flies were placed in vials at 25°C, 20 per vial, and with food media made of 1% sucrose, 1% dry yeast extract, 1.2% agar (w/v), and 1.5% hydrogen peroxide (Camming et al. 2008). In addition to the ROS exposed set, a control set using the same conditions in the absence of hydrogen peroxide was done for each genotype. Mortality was scored every 12 hr and the media was replaced every 24 hr.
Flight assay

Flight assays were conducted according to previously published protocols (Kawasaki et al. 2016). Briefly, adult flies are introduced into the top of a large diameter cylinder coated with oil. Flies stick to the wall of the cylinder when they land and the height of their landing provides a measure of their capacity to maintain flight; flightless animals fall to a pool of oil at the bottom of the cylinder.

Immunohistochemistry and confocal analysis

Whole-mount immunostaining of adult flight muscles was carried out according to described procedures to visualize both ubiquitin levels and the organization of actin filaments (Hunt and Demontis 2013). Images were acquired at room temperature using an Olympus Fluoview FV1000 laser-scanning confocal microscope (Olympus America, Waltham, Massachusetts, USA). FV10-ASW 2.1 software (Olympus, Waltham, Massachusetts, USA) was used to capture images. When more than one fluorophore was detected, sequential line scanning was performed to avoid spectral bleed through artifacts. Images of samples with different genotypes within a single experiment were captured, processed, and analyzed using the same settings. Images were presented as Z-stacks of maximum intensity projections using Imaris 7.3 software (Bitplane Inc.). All adjustments to contrast and brightness were made to ease interpretation of confocal images applied identically to all genotypes within each experiment.

Image analysis of ubiquitin-positive intracellular punctae in flight muscles

ImageJ was used to identify and measure the number of anti-ubiquitin antibody-positive punctae in the adult flight muscles of $sfl$ park $\Delta 21$/mef2-Gal4 park1 and UAS-Atg5RNAi/+; $sfl$ park $\Delta 21$/mef2-Gal4 park1 animals. Animals were reared at 25°C for the experiments illustrated in Figure 9. The presence of the UAS-Atg5RNAi adversely affected survival of $sfl$ park $\Delta 21$/mef2-Gal4 park1 (less than 10% of expected, n = 733). For every image, the brightness of the color threshold was set to a minimum of 70, to identify the punctae over background. The “Measure” function was used to determine the area and the “Particles” function was used to count the number of particles, with the circularity of the particles set to the restraints of 0.80–1.00. The “Exclude on Edges” function was enabled to remove extraneous staining at the edges of the preparation, and the number of punctae per unit area was determined. Differences between punctae/area for the genotypes was assessed using a two-tailed t-test. A replicate experiment conducted at a lower temperature (23°C) for the genotypes was assessed using a two-tailed t-test.

Insoluble ubiquitin protein assay

A two-step protein extraction was used for Insoluble Ubiquitin Protein analysis, modified from a previously described method (Cumming et al. 2008). Typically, 30-60 adult heads were homogenized by ceramic bead agitation using the bead Ruptor 24 (Omni International, Kennesaw, Georgia, 73 USA) in 100μl of SDS extraction buffer (2% SDS, 50mM Tris pH 7.4, 1X protease inhibitor cocktail Complete [Roche, 10184600]). Samples were spun down at 10,000rpm for 10 min and the supernatant was removed to a clean tube.

Western blotting

IUP assessment utilized 9% acrylamide gels, while the analysis of Mcherry-Atg8a utilized 6% acrylamide gels. PAGE was performed using the BioRad mini-PROTEAN electrophoretic and transfer system (BioRad, Hercules CA) with 1 mm plates. Protein samples were prepared for loading and membranes were processed as described above. Primary antibody incubation was performed overnight at 4°C using 1:2000 mouse anti-Mono- and Polyubiquitinylated Conjugates (FK2) (Enzo, BML-PW8810-0100) or 1:2000 rabbit anti-mCherry (abcam, ab183628), and 1:3000 mouse anti-tubulin (Developmental Studies Hybridoma Bank, 12G10). Secondary antibody incubation (1:3000 HRP conjugated goat anti-mouse or –rabbit, Invitrogen, 31430 and 7431460) was performed for 45 min at room temperature. ECL detection was performed using G:BOX Chemi XG4 (Syngene, Fredrick, Maryland, USA) which was also used to invert coloration of resulting images. Densitometry was
performed using ImageJ 1.42q. For loading analysis, a combination of Ponceau staining and stripping and reprobing with anti-alpha tubulin was used. Membranes were stripped for re-probing in mild stripping buffer (0.1% SDS, 1.0% Tween20, 0.2M glycine, pH 2.2). All blotting experiments were performed using three biological replicates for each genotype under each condition. Lanes with the most numerically similar sample loading, as determined by densitometry, were chosen from within the biological triplicates for display.

**Quantitative reverse transcription PCR**

QPCR assays of sfl, ttv and parkin were carried out to evaluate the levels of these transcripts in animals heterozygous for sfl or ttv alleles and either heterozygous or homozygous for park alleles. Transcript levels were also measured in larvae bearing UAS-sfRNAi or UAS-ttRNAi expressed under the control da-Gal4.

RNA was isolated from 20 flies per sample with a Macherey-Nagel DNA, RNA and Protein Purification Kit. RNA was diluted to 200 ng/μl then used in a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). This was put through an Eppendorf Mastercycler Pro PCR machine to create cDNA. Real time PCR was performed in a StepOne machine, using TaqMan assays for Drosophila gene targets park, sfl, ttv, and arginine kinase (Thermo Fisher Scientific).

Data were analyzed using Thermo Fisher Connect software to determine the relative quantifications. Three biological replicates with two technical replicates were used for each genotype. Target gene data were normalized to arginine kinase using the ΔΔCt method.

**Statistical analysis**

Statistical analyses of quantitative data were performed using either Minitab Release 16 (Minitab) or GraphPad Prism 8.1.2 (GraphPad Software). Data were represented as the mean ±95% confidence interval unless otherwise indicated. Data distributions were tested using D’Agostino &amp;amp;amp; Pearson normality test. Comparisons between more than two groups were performed using ANOVA or Kruskal-Wallis for nonparametric and or heteroscedastic data, followed by individual pairwise comparisons using Dunn’s test for non-parametric data or a t-test for normally distributed data.

**Data availability**

Strains and plasmids are available upon request. All other data necessary for confirming conclusions of the study are present with the article, figures and table. Supplemental material that includes, Table S1: Analysis of heparan sulfate levels and structure in sfl and ttv heterozygotes, Figure S1: Frequency distribution of pixel intensities for anti-ubiquitin staining in flight muscles of parkin mutants and ttv/+; park^{231}/park^{1}animals, Figure S2: QPCR analysis, Figure S3: Original western blots used for assembly of Figure 4, are available at figshare: https://doi.org/10.25387/g3.9989801.

**RESULTS**

**RNAi of genes required for heparan sulfate biosynthesis increase autophagy-dependent cleavage of Atg8a in the brain**

Earlier work demonstrated that reducing heparan sulfate biosynthesis increased autophagy in muscle and fat body, the latter being the principle metabolic sensing and energy storage tissue in Drosophila (Reynolds-Peterson et al. 2017). Given the critical role autophagy plays in neurodegenerative processes we wanted to determine directly if heparan sulfate biosynthesis affects autophagy in the brain. Autophagy-mediated proteolysis produces cleavage of key components, including Atg8a, a protein involved in autophagosome formation. Monitoring Atg8a cleavage is therefore one measure of autophagy. To evaluate Atg8a proteolytic processing we expressed an mCherry-Atg8a fusion protein in the brain using a UAS-mCherry-Atg8a fusion protein in the brain using a UAS-mCherry-Atg8a construct and a neuron-specific Gal4 transcriptional activator. The resistance of the mCherry domain of the fusion protein to degradation allows the visualization of a relatively stable proteolytic product detected with an anti-mCherry antibody. The ratio of the parental protein, mCherry-Atg8a, to the mCherry proteolytic product provides a measure of autophagy-dependent activity (Figure 1)(Klionsky et al. 2016). As previously reported, activation of autophagy by transgene expression of Atg8a produced greater conversion of the mCherry-Atg8a fusion protein to the smaller mCherry-containing fragment. We examined the effect of heparan sulfate structure on Atg8a proteolytic cleavage by knockdown of either of two genes required for heparan sulfate biosynthesis, sulfatless (sfl) or tout velu (ttv). sfl, encodes N-deacetylase N-sulfotransferase, an enzyme affecting sulfation of the heparan sulfate polymer (Toyoda et al. 2000a; Toyoda et al. 2000b). ttv encodes a homolog of Exostosin 1 (Ext1), a glycosyl transferase critical for heparan sulfate chain elongation. RNAi of either sfl or ttv promoted proteolytic cleavage of mCherry-Atg8a, indicative of a net increase in autophagy in the CNS (Figure 1).

**Reductions of heparan sulfate biosynthetic gene function increase lifespan**

Increases in autophagy can extend lifespan, and longevity therefore provides a measure of autophagy function in the organism (Kapahi et al. 2017; Kinghorn et al. 2016; Wang et al. 2016). Lifespan was evaluated in adult animals heterozygous for mutations in sfl or ttv, each affecting different components of the heparan sulfate biosynthetic apparatus.
Reducing the function of either of these two genes provided significant extension of lifespan compared to wild type control animals in both males and females (Figure 2). Overall, ttv/+ appears to increase lifespan to a greater degree than sfl/+ animals. We do not know the basis of these differences but heterozygosity for mutations in these genes does affect the levels and structure of the heparan sulfate polymer differently (see below and Supplemental Table S1), perhaps accounting for the differential effects on lifespan. Together, these data provide evidence of the systemic impact of heparan sulfate modified proteins on the physiology of the organism consistent with an increase in autophagy levels.

Given the effect of heterozygosity for genes required for heparan sulfate biosynthesis on lifespan it was of interest to determine the structure and levels of the heparan sulfate polymer in adult animals bearing these genotypes. Disaccharide profiling of heparan sulfate derived from ttv/+ and sfl/+ animals compared to wild type controls was conducted using a method that provides both levels and composition of six disaccharides that comprise the polymer (Toyoda et al. 2000a; Toyoda et al. 1999). These analyses demonstrated that reducing the gene function by approximately 50%, with heterozygosity for alleles of either sfl or ttv, had a detectable and significant effect on both the quantity and sulfation state of the heparan sulfate polymer. In two different and independently isolated sfl alleles, heterozygosity lowered the levels of heparan sulfate-derived disaccharides by approximately 20% (Table S1) and also altered the sulfation pattern, reflected in the levels of different mono, bi and tri-sulfated disaccharides. Two disaccharides, the monosulfated NS(ΔUA-GlcNS), and the disulfated 2SNS (ΔUA2S-GlcNS), were affected the most, reduced by approximately 20% compared to wild type animals. Animals heterozygous for a ttv null allele, showed lower amounts of all disaccharides, to levels between 65–79% of wild type but without remarkable changes in the sulfation pattern. Previous analysis of third instar larvae heterozygous for ttv showed similar effects (Toyoda et al. 2000a). These results are consistent with the established activities encoded by sfl and ttv.

Reducing heparan sulfate biosynthetic capacity increases resistance to ROS stress

Increasing autophagy provides protection against exposure to reactive oxygen species (ROS) stress by elevating the capacity of cells to remove...
damaged macromolecules. Given the broad increases in autophagy measured in whole larvae, larval muscle and fat body, as well as adult brain upon reduction of heparan sulfate biosynthesis we wanted to determine if organism-wide decreases in heparan sulfate gene function could affect responses to oxidative stress. Measures of resistance to ROS exposure were therefore conducted for animals heterozygous for mutant alleles of sfl, itv, and brother of itv (botv). botv encodes the homolog to vertebrate EXT Like-3, an N-acetylgallosamine transferase-II required for heparan sulfate synthesis. To examine ROS sensitivity, adult flies were continuously exposed to H_{2}O_{2} in food media and their survival monitored. Reductions in heparan sulfate biosynthetic gene function significantly increased survival to ROS exposure for all animals heterozygous for mutations in either of these three heparan sulfate biosynthetic enzyme-encoding genes (Figure 3). In female animals, wild type controls had a median survival duration of 48 hr, while sfl^{O84/+} or sfl^{O84/+} animals survived for a median of 72 and 96 hr respectively. Heterozygosity for itv further extended median survival to 120 hr, while botv/+ animals survived for a median of 72h. Males were more sensitive to peroxide exposure than females, but heterozygosity for all of these alleles still significantly increased their tolerance. These findings indicate that compromising heparan sulfate gene function can have a significant impact on a whole-organism response to ROS.

**Knockdown of heparan sulfate biosynthetic enzyme encoding genes in the brain reduced levels of insoluble ubiquitin-modified proteins upon ROS exposure**

Ubiquitin-modified proteins are subject to targeted autophagic degradation through binding to the autophagy receptor p62. Particularly in mature adults, levels of autophagy in a tissue are generally inversely proportional to the amount of ubiquitin present. We assessed the levels of insoluble ubiquitin-modified proteins isolated from the brains of adult animals with CNS-directed RNAi of sfl or itv after a 24-hour exposure to control or H_{2}O_{2}-containing food. Adult heads were isolated, the protein solubilized, and the Triton X-100 insoluble fraction obtained according to published protocols (Simonsen et al. 2008). This fraction was separated by SDS-PAGE and ubiquitin-modified proteins detected by western blotting. This procedure has been used to detect age- and autophagy-dependent changes in the clearance of ubiquitin-modified substrates in the CNS of adult Drosophila. The levels of ubiquitin were measured for the entire lane for each sample and the levels normalized by comparison to the signal detected with anti-tubulin antibody. In control animals, ROS exposure increased the level of ubiquitin-modification of brain proteins, while increasing autophagy by over-expression of Atg8a reduced the levels of ubiquitin-modified proteins compared to control animals (Figure 4). In adult flies expressing either of two unique RNAi constructs to knock down sfl expression, the levels of insoluble ubiquitin-modified proteins were similarly reduced in oxidant-exposed animals compared to controls. These findings show that the level of key genes required for heparan sulfate biosynthesis have an impact on ROS-mediated accumulation of ubiquitin-modified proteins in the brain.

**Knockdown of sfl or itv suppresses neurodegeneration mediated by overexpression of Presenilin**

Reducing heparan sulfate biosynthetic function provided protection from ROS, extending survival to peroxide exposure. This enhanced survival was accompanied by reductions in ubiquitin-modified proteins in the brain, indicative of increased clearance of damaged proteins. We therefore wanted to determine if reductions of heparan sulfate biosynthesis were protective for neurotoxic stress. Missense mutations in Presenilins account for a sizable fraction of familial AD cases and models of Presenilin-mediated neurodegeneration have been established in Drosophila. Recent analysis of 138 pathogenic mutations in PSEN1 demonstrate that approximately 90% compromise the function of the encoded γ-secretase (Sun et al. 2017) further supporting the hypothesis that neuronal loss is likely a consequence of this reduced function (Kelleher and Shen 2017). Overexpression of Presenilin (Psn) in Drosophila produces apopotic and neurogenic phenotypes resembling Presenilin loss-of-function phenotypes, indicating this model provides some parallels with the human pathology (Ye and Fortini 1999). Presenilin overexpression in the Drosophila retina produces neuronal loss and patterning abnormalities. We have employed this model to determine if downregulation of heparan sulfate biosynthesis could affect neuronal loss and disruption of retinal patterning mediated by overexpression of Psn. A Drosophila Psn transgene was expressed under the direction of a neuron-specific Gal4 line, in the presence or absence of UAS- transgenes encoding double-stranded RNAi targeting either itv or sfl mRNAs. Expression of Psn produces a marked reduction in the size of the adult eye as well as a disruption of patterning, seen in the disorganization of the facets of the retina (Figure 5, compare A and B). RNAi of sfl produced a significant rescue of both retinal size reduction and disordering (Figure 5, A,B,D, E and F). The degree of patterning defect was measured using Flynotyper (Iyer et al. 2016), an automated image processing algorithm that measures several features of retinal organization using the light reflected from each eye facet. Flynotyper
calculates a single numerical score, reflecting several features of eye geometry. RNAi of ittv significantly rescued retinal size (Figure 5, panel C,E), represented as eye area, but did not improve the degree of retinal disorganization (Figure 5 F). Thus, compromising the function of two different genes in the heparan sulfate biosynthetic pathway reduced the developmental toxicity of Psn overexpression.

**Altering heparan sulfate structure rescues muscle degenerative phenotypes of parkin mutants**

Autophagy is responsible for engulfment and removal of damaged mitochondria, a function critical for mitochondrial quality control. Mutations in parkin (park), a Drosophila homolog of the human Parkin-encoding gene (PARK2), have been identified and animals bearing these mutations characterized (Burman et al. 2012; Pesah et al. 2004). Mutations in the human gene, PARK2, are responsible for a substantial fraction of familial Parkinson Disease patients, emphasizing the relevance of understanding PARK2/parkin function in the pathophysiology of this disorder (Giannoccaro et al. 2017; McWilliams and Muqit 2017). Studies of Drosophila park mutants have demonstrated that Park affects mitochondrial surveillance and autophagy-mediated removal (Pesah et al. 2004; Vincow et al. 2013). Loss of park produces degeneration of flight muscle, a highly metabolically active cell, and certain allelic combinations of park (park¹/park²) survivors to adulthood but are flightless, showing progressive muscle degeneration (Pesah et al. 2004). We examined the capacity of altering heparan sulfate biosynthesis to alter flight muscle cell degeneration in park mutants with the rationale that increasing autophagy could ameliorate the accumulation of damaged mitochondria. Heterozygosity for sff had a profound effect and reduced the severity of park mutant phenotypes (Figure 6). Both sff⁰⁰⁰⁰⁰⁰⁰⁰ and sff³⁸⁴⁴ alleles had similar effects on suppressing muscle cell abnormalities, including the accumulation of ubiquitin-modified proteins and disordering of actin filaments (Figure 6 A-C). Muscle function was evaluated with an assay that provides a quantitative assessment of flight. park¹/park² animals are incapable of flight (zero score) whereas sff⁰⁰⁰⁰⁰⁰⁰⁰/park² flies have near wild type levels (Figure 6D). Similar analyses were conducted for interactions between ittv and park and heterozygosity for a null allele of ittv also showed significant rescue of park¹/park² flight muscle abnormalities (Figure 7). Reduction of ittv function also reduced the levels of ubiquitin-modified proteins in the indirect flight muscle of park mutants (Figure 7 and Figure S1). Quantitative PCR was used to confirm that mutations in sff, ittv and park (park²) reduced the respective levels of mRNA (Figure S2) and predicted levels of mRNA were found in the animals that showed rescue of park-mediated phenotypes. The rescue of park mutant animals by reductions of sff or ittv function provides evidence of the capacity of altered heparan sulfate synthesis to promote cellular repair systems that counteract cellular stresses, including accumulation of damaged mitochondria.

**Figure 5** Reduction of sff or ittv function rescues retinal abnormalities produced by overexpression of Presenilin. Transgene-mediated overexpression of Presenilin in neurons produces cell death and a reduction of the size of the eye, as well as disrupted patterning. Using brightfield illumination, serial optical sectioning and computational reconstruction, high-resolution images of the retina were obtained. Light reflection from each ommatidium (the photoreceptive unit) provides the location and hence geometry of the retina. Expression of Presenilin (elav-Gal4::155 > UAS-Psn) produces a rough and reduced retina (compare panels A and B; 9 animals for control, 10 animals for each experimental genotype), as well as disruption of the arrangement of ommatidia. The area of the retina provided a measure of cell loss (E). A computational method (Flynotyper) was used to obtain a measure of retinal disorganization, with higher scores representing increased disarray (F). RNAi of either sff or ittv suppressed the effects of Presenilin overexpression on retinal size to a significant degree (compare panels B to C and D and measurements, panel E), whereas only sff RNAi significantly rescued the disordering of the retina, measured by Flynotyper morphological scoring parameters (panel F). In panels E and F, data are presented as a scatter plot of each animal with the mean plus/minus 95% confidence interval. * (top row) indicate comparison to control, while + (bottom row) indicate comparison to Presenilin expression alone. /+ P < 0.05, **/++ P < 0.01, ***/+++ P < 0.001, **** P < 0.0001. Statistical testing utilized Kruskal-Wallis non-parametric group test followed by Dunn’s pairwise comparisons.
Parkin functions in the surveillance and tagging of damaged mitochondria for removal by autophagic degradation. This is achieved via Parkin mediated ubiquitin-modification of outer membrane mitochondrial proteins, providing a molecular tag for recognition by the autophagy machinery. In accordance with the function of Parkin in mitochondrial surveillance, parkin mutants show accumulations of abnormal mitochondria. To determine if this critical and central phenotype of parkin mutants is affected by the levels sfl function we examined animals where mitochondrial were selective tagged by the expression of mito-GFP, a mitochondrial targeted protein, under the direction of a muscle-specific Gal4 line, mef2-Gal4. parkin mutants showed large and dysmorphic mitochondria compared to control animals and these changes were reversed by heterozygosity for sfl (Figure 8). These results establish that changes in heparan sulfate structure can modulate the cellular pathology of parkin mutants at the level of the primary deficit, failure to tag and removed damaged mitochondria.

**Autophagy dependence of sfl-mediated rescue of cell degeneration in parkin mutants:** Alteration of heparan sulfate structure has a dramatic impact in two genetic models of cell degeneration in Drosophila, overexpression of Presenilin and parkin mutants. It is also evident that heparan sulfate structure can modulate autophagy in muscle, fat body, and neurons (Reynolds-Peterson et al. 2017) [data presented in this study]. To determine if autophagy function is required for the capacity of sfl to affect the rescue of cell degeneration in parkin mutants we used muscle-directed expression (mef2-Gal4) of Atg3RNAi. In parkin mutant animals ubiquitin accumulation is reduced by the presence of a single sfl mutant allele (Figure 6). When reared at 25°C, a temperature where Gal4-directed transcription is active, RNAi of Atg3 resulted in significantly increased accumulation of ubiquitin in the muscle cells of sfl park1/mef2-Gal4 park1 adult animals (Figure 9). At this temperature, the knockdown of Atg3 produced significant lethality in sfl park1/mef2-Gal4 park1 animals. A replicate experiment at 23°C, where Gal4 activity, and hence the degree of RNAi, was lower, reduced the lethality and also showed significant increases in ubiquitin-positive punctae in Atg3RNAi bearing animals, although the punctae were fewer and less bright (t-test, P = 0.01). The elevated levels of punctae required both parkin alleles to be present. These findings demonstrate that an intact autophagy system is required for the full rescue of parkin mutants mediated by reductions in sfl function.

**DISCUSSION**

**Heparan sulfate biosynthesis levels and the regulation of autophagy**

Earlier work established that compromising heparan sulfate biosynthesis in Drosophila produces elevated levels of autophagy in larval muscle and fat body, the latter being a critical energy storage and metabolic sensing organ (Reynolds-Peterson et al. 2017). In studies presented here, we examined if heparan sulfate modified proteins have a broader, organism-wide role in regulating autophagy, and affect physiological processes known to be autophagy-dependent. We also examined heparan sulfate-dependent autophagy in the adult brain using two assays, autophagy-dependent cleavage of Atg8a, a critical autophagy component, as well as the levels of insoluble ubiquitin-modified proteins (IUPs) in the adult brain after ROS exposure. IUPs show age-dependent accumulation and levels of these proteins are affected by both autophagy function and exposure to ROS (Simonsen et al. 2008). In both of these experimental systems, reducing heparan sulfate biosynthesis showed the hallmarks of increased autophagy, elevated cleavage of Atg8a and reduced levels of IUP with ROS treatment. These findings demonstrate the capacity of heparan sulfate levels to affect autophagy in the brain and...
heparan sulfate structure to a modest degree that does not compromise some of the vital functions of heparan sulfate modified proteins and allows the beneficial effects of removing inhibitory activities on cellular processes, such as increased autophagy, to become apparent. In short, these pathways, both heparan sulfate biosynthesis and autophagy, are not all or none switches but can have very different effects depending on their level of function.

**Heparan sulfate biosynthesis effects on lifespan and resistance to oxidative stress**

Autophagy has profound consequences on the physiology of the organism as a whole. Increased autophagy is associated with extended lifespan in a number of model organisms, including yeast, *C. elegans*, *Drosophila* and mice (Kapahi et al. 2017; Simonsen et al. 2008; Hansen et al. 2018; Fernández et al. 2018). Expression of autophagy genes and proteins show age-dependent reduction in virtually all model organisms examined. In humans, mutations affecting autophagy components are associated with a number of disorders affecting cell health and viability, suggesting that the functional connection between autophagy, ageing and age-dependent diseases is conserved (Hansen et al. 2018; Yan and Klionsky 2008; Fernández et al. 2018). Given the effect of elevated autophagy on ageing we examined the lifespan of fruit flies heterozygous for genes encoding critical enzymes of heparan sulfate biosynthesis and sulfation. Reducing the function of *ttv* or *sfl* all increased lifespan significantly, providing evidence that compromising processes supported by heparan sulfate modified proteins was protective to a physiologically relevant degree and consistent with broadly elevated levels of autophagy.

ROS can contribute to cell damage and molecular systems designed to remove these molecules provide important cell protective mechanisms. Autophagy has a number of roles in mitigating oxidative stress. First, a principal source of ROS, mitochondria, is removed by an autophagy-dependent mechanism, mitophagy. In particular, damaged mitochondria, that produce higher levels of ROS, are tagged for removal and lysosomal degradation mediated by autophagocytosis. Autophagy is also able to remove damaged proteins and protein aggregates that can result from ROS damage. It therefore follows that increases in autophagy can provide a level of resistance to ROS exposure, and this has been demonstrated experimentally. In *Drosophila* adding hydrogen peroxide to food media is lethal over a several day course and can be used to assess sensitivity to oxidative stress. Reductions in heparan sulfate biosynthesis mediated by heterozygosity for mutations in any of three genes significantly increased survival upon exposure to hydrogen peroxide, consistent with protection afforded by increases in autophagy.

**Figure 7** Reduction of *ttv* function rescues flight muscle abnormalities of *parkin* mutant animals. Adult flight muscles were dissected, fixed, stained with anti-ubiquitin antibody and visualized by confocal microscopy. *park<sup>d21</sup>/+* animals served as controls for this experiment. The frequency distribution of anti-ubiquitin signal intensity in animals bearing a single allele of *ttv* (panels B vs. C). The frequency distribution of anti-ubiquitin signal intensity level, see Supplemental Figure 2) was assessed for 15 animals of each genotype and analyzed using the Wilcoxon Signed Ranked Median Test. This assessment showed a significant difference (*P* < 0.005) between *park<sup>d21</sup>/park<sup>1</sup>* and *ttv<sup>20681</sup>; park<sup>d21</sup>/park<sup>1</sup>* animals. Scale bars represent 200μM.

**Figure 8** Reducing *sfl* function rescued mitochondrial abnormalities of *parkin* mutants. Mitochondria in adult flight muscle cells were tagged with UAS-mitoGFP expressed in muscle under the direction of a muscle-specific Ga<sup>4</sup> line, mel2-Ga<sup>4</sup>. This marker was crossed into *parkin* mutants and *parkin* mutants bearing a single mutant allele of *sfl*. Control animals (A) are wild type genotype, and B and C show *parkin* mutants without and with the *sfl* allele respectively. Representative images from two separate experiments are shown (n = 52). The scale bar represents 30μM.
This is strong evidence that modulation of heparan sulfate synthesis has a broad impact on autophagy and physiology governed by this important cellular process.

Resistance to ROS stress was achieved in animals heterozygous for mutations in key heparan sulfate biosynthetic enzyme-encoding genes. Complete loss of function of these genes is lethal, affecting signaling mediated by many essential patterning molecules, including Wingless, a broad impact on autophagy and physiology governed by this important cellular process. Target of Rapamycin (TOR) is a master regulator of autophagy, affecting both the activity of key Atg gene transcription factors as well as the phosphorylation of cells. Previous work has shown that reductions of heparan sulfate biosynthesis or sulfation in muscle cells produces a reduction in mitochondrial density. Autophagosomes surrounding mitochondria were readily visualized in animals with compromised heparan sulfate biosynthesis, supporting the model that increased mitophagy is taking place (Reynolds-Peterson et al. 2017). These findings suggested that heparan sulfate-mediated regulation of autophagy can affect mitophagy. Therefore, we sought to determine if reducing heparan sulfate-biosynthesis can affect cellular loss mediated by defects in mitochondrial surveillance and removal. parkin is the Drosophila homolog of PARK2, a human gene responsible for a form of juvenile onset Parkinson’s disease (Pesah et al. 2004). Park protein is a ubiquitin ligase responsible for the tagging of damaged mitochondria for autophagosome-lysosomal destruction. Loss of park results in age-dependent degeneration of Drosophila flight muscles, with associated accumulation of dysmorphic mitochondria (Cackovic et al. 2018; Cornelissen et al. 2018; Vincow et al. 2013). Reducing the gene dosage of sfi had a remarkable capacity to rescue muscle cell death mediated by park mutations, affecting actin morphology, accumulation of ubiquitin, and dysmorphism of mitochondria as well as the key measure of flight muscle function, the capacity to fly. Reductions of ttk function also rescued muscle abnormalities of park mutants. These in vivo interactions demonstrate the capacity of heparan sulfate levels and structure to rescue cell degeneration in a model of a human neurodegenerative disorder.

The ability of reductions in sfi function to rescue muscle abnormalities in parkin mutants was dependent on an intact autophagy system (Figure 9). RNAs of a key autophagy gene, Atg5, increased ubiquitin-positive accumulations in muscle, a characteristic of parkin mutants. These findings support the model that changes in heparan sulfate structure affect cell survival via alterations in autophagy levels. However, changes in heparan sulfate may be affecting other biological responses that have an impact on cell physiology in parkin mutants; our data indicate autophagy is an important part of that response.

**Mechanism of heparan sulfate-mediated regulation of autophagy**

Regulation of autophagy is complex and includes transcriptional, post-transcriptional and post-translational mechanisms (Feng et al. 2015). Target of Rapamycin (TOR) is a master regulator of autophagy, affecting both the activity of key Atg gene transcription factors as well as the activity of autophagosome assembly proteins. Tor activity serves to suppress autophagy and growth conditions that favor Tor activation result in reduced levels of autophagic flux. Heparan sulfate modified proteins play critical roles in signaling pathways that lead to Tor activation via PI3 kinase and this is one potential mechanism of heparan sulfate-mediated regulation of autophagy.
sulfate-mediated regulation of autophagy. Autophagy can also be activated by accumulation of unfolded proteins and the subsequent unfolded response (UPR) cascade. Earlier we examined one element of the UPR, the IRE-mediated splicing of Xbp (Ryoo et al. 2007) and found that reductions of heparan sulfate biosynthesis had no effect on this measure of the UPR. In addition, third instar larval muscles from animals bearing ttv mutations show lowered levels of BiP (Ren et al. 2009), a protein that is typically elevated upon unfolded stress responses (Pobre et al. 2018). Interestingly, sfi or ttv mutant larvae also show increased levels of stimulus-dependent endocytosis in the motoneuron, emphasizing that the effects of heparan sulfate structure on membrane trafficking are not limited to autophagy (Ren et al. 2009).

This work documents the broad inhibitory effect of heparan sulfate modification on autophagy in Drosophila. Studies in mice suggest this regulatory relationship is represented in vertebrates as well. Heparanase (Hpa) is an endo-D-glucuronidase that cleaves heparan sulfate and transgenic mice with ectopic expression of Hpa exhibit increased autophagy levels in multiple tissues. Conversely, knockouts of Hpa results in autophagic suppression (Ilan et al. 2015; Shteingauz et al. 2015). Collectively, these findings suggest heparan sulfate modulated protein suppression of autophagy is evolutionarily conserved and occurs in many cell types and tissues in mice.

**Implications of heparan sulfate-mediated regulation of autophagy in human neurodegenerative disorders**

The capacity of autophagy to remove protein aggregates and damaged mitochondria provides a means of protecting cells against pathological events that lead to cell death (Gil and Rego 2008; Komatsu and Puche 2006a; Ramesh and Pandey 2017; Wagner and Russell 2017). Activation of autophagy has the potential to be protective for neurodegeneration and conversely, suppression of autophagy confers susceptibility to cell loss. Interestingly, lysosomal storage disorders, where deficits in certain degradative enzymes result in accumulation of heparan sulfate, produce suppression of autophagy and neuronal loss (Bartolomeo et al. 2017; Fiorenza et al. 2018; Settembre et al. 2008a; Settembre et al. 2008b). A number of human neurodegenerative disorders, including AD, Huntington’s, and Amyotrophic Lateral Sclerosis, show protein aggregation and accumulation. Deficits in mitochondrial turnover and clearance are also implicated in neurodegeneration. Parkinson’s Disease being a well-studied case in point (Buhlman 2017; Nakamura et al. 2013; Trempe and Fon 2013). The data presented here demonstrates that modulation of heparan sulfate synthesis has the capacity to rescue cell loss in two models of human neurodegenerative diseases, one mediated by overexpression of Presenilin and the other by loss of parkin function. Overexpression of Presenilin phenocopies loss-of-function mutants in Drosophila suggesting this model works via a dominant-negative mechanism. Given that the majority of AD pathogenic mutations affecting PSEN1 reduce or eliminate γ-secretase function, the fly model may provide a reasonable assay for Presenilin-mediated pathology. Other experiments described here also show that reducing heparan sulfate biosynthetic capacity can rescue cell pathology produced by reductions of parkin function. Parkin, the fly homolog of PARK2, participates in mitochondrial surveillance via the ubiquitin-modification of outer membrane proteins, tagging them for mitophagy and degradation in the lysosome. Our experiments show that reducing heparan sulfate can suppress the muscle abnormalities in parkin mutants, including the restoration of mitochondrial morphology, and suggests that deficits in mitochondrial surveillance can be rescued by, increasing autophagy. Heparan sulfate biosynthesis may well be a useful target for intervention to increase autophagy and mitophagy, providing some protection in a variety of neurodegenerative disorders.

Earlier work has implicated heparan sulfate modified proteins in the deposition or clearance of amyloid deposits in the brain. Two studies support the proposal that reducing heparan sulfate decreases amyloid deposition in mouse models of AD. Conditional knockout of Ext3 in post-natal neurons dramatically reduced the levels of amyloid plaques in an APP/PS1 mouse model (Liu et al. 2016). Similarly, overexpression of heparanase, a heparan sulfate degradative enzyme, also reduced amyloid burden in a mouse AD model (Jendresen et al. 2015). Heparan sulfate modified proteins are found in amyloid plaques and may play a significant role in both amyloid β deposition or clearance. Our findings suggest reduction in heparan sulfate modification could also affect autophagy, which does show alterations in AD. Autophagy is impaired in AD (Nixon and Yang 2011) and mechanisms that restore it to normal levels could be protective. Certainly, the capacity of heparan sulfate modified proteins to suppress autophagy has implications for understanding how reductions in the levels of heparan sulfate modification affect processes leading to neurodegeneration.

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