Ion-current-based Proteomic Profiling of the Retina in a Rat Model of Smith-Lemli-Opitz Syndrome*

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Smith-Lemli-Opitz syndrome (SLOS) is one of the most common recessive human disorders and is characterized by multiple congenital malformations as well as neurosensory and cognitive abnormalities. A rat model of SLOS has been developed that exhibits progressive retinal degeneration and visual dysfunction; however, the molecular events underlying the degeneration and dysfunction remain poorly understood. Here, we employed a well-controlled, ion-current-based approach to compare retinas from the SLOS rat model to retinas from age- and sex-matched control rats ($n = 5$/group). Retinas were subjected to detergent extraction and subsequent precipitation and on-pellet-digestion procedures and then were analyzed on a long, heated column (75 cm, with small particles) with a 7-h gradient. The high analytical reproducibility of the overall proteomics procedure enabled reliable expression profiling. In total, 1,259 unique protein groups, $\sim$40% of which were membrane proteins, were quantified under highly stringent criteria, including a peptide false discovery rate of 0.4%, with high quality ion-current data (e.g. signal-to-noise ratio $\geq 10$) obtained independently from at least two unique peptides for each protein. The ion-current-based strategy showed greater quantitative accuracy and reproducibility over a parallel spectral counting analysis. Statistically significant alterations of 101 proteins were observed; these proteins are implicated in a variety of biological processes, including lipid metabolism, oxidative stress, cell death, proteolysis, visual transduction, and vesicular/membrane transport, consistent with the features of the associated retinal degeneration in the SLOS model. Selected targets were further validated by Western blot analysis and correlative immunohistochemistry. Importantly, although photoreceptor cell death was validated by TUNEL analysis, Western blot and immunohistochemical analyses suggested a caspase-3-independent pathway. In total, these results provide compelling new evidence implicating molecular changes beyond the initial defect in cholesterol biosynthesis in this retinal degeneration model, and they might have broader implications with respect to the pathobiological mechanism underlying SLOS. 

Molecular & Cellular Proteomics 12: 10.1074/mcp.M113.027847, 3583–3598, 2013.

Smith-Lemli-Opitz syndrome (SLOS)$^1$ is an autosomal recessive disorder associated with subnormal growth and failure to thrive, mental retardation and neurosensory deficits, and multiple congenital anomalies, including dysmorphologies (1, 2). Early epidemiological studies estimated the incidence of SLOS as 1 in 20,000 to 1 in 60,000 live births, primarily among Caucasians (1, 2). However, more recent studies suggest that the SLOS carrier frequency is $\sim$1 in 30 to 1 in 50; this predicts a much higher actual disease frequency, ranging from 1 in 1,590 to 1 in 17,000 (3, 4), making SLOS the fourth most common autosomal recessive human disease (after cystic fibrosis, phenylketonuria, and hemochromatosis). Mutation of the $DHCR7$ gene is the intrinsic cause of SLOS; this gene encodes the enzyme $DHCR7$ (3$\beta$-hydroxycholesterol-$\Delta$I-reductase, a.k.a. 7-dehydrocholesterol reductase; EC1.3.1.21), which catalyzes the final step in the cholesterol biosynthetic pathway, reducing the $\Delta$I double bond and thus converting 7-dehydrocholesterol (7DHC) to cholesterol (4, 5). As a consequence, markedly reduced levels of cholesterol and aberrantly elevated levels of the cholesterol precursor 7DHC (and its epimer, 8DHC) are observed in the majority of affected SLOS patients (6, 7). Therefore, the clinical suspicion of SLOS

$^1$ The abbreviations used are: ApoA, apolipoprotein A; ApoE, apolipoprotein E; AUC, area under the curve; CATD, cathepsin D; cSREBP, cleaved sterol regulatory element-binding protein; DHC, dehydrocholesterol; GFAP, glial fibrillary acidic protein; ONL, outer nuclear layer; RPE, retinal pigment epithelium; SLOS, Smith-Lemli-Opitz syndrome; SREBP, sterol regulatory element-binding protein; Stat3, signal transducer and activator of transcription 3; TBS, Tris-buffered saline; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.
is confirmed by elevated 7DHC in plasma or tissues, typically demonstrated via chromatographic methods (e.g. HPLC or GC/MS) (8, 9).

Visual capacity may become compromised in SLOS patients because of a variety of congenital or postnatal pathologies, such as cataracts, aniridia, corneal endotheliopathy, sclerocornea, electroretinographic defects in the retina, optic nerve abnormalities, or other ophthalmologic problems (10, 11). We currently lack full knowledge of the exact pathobiological mechanism underlying SLOS, but additional insights may be afforded by studies employing a rodent model of the disease in which rats are treated with AY9944 (trans-1,4-bis[2-chlorobenzylaminomethyl] cyclohexane dihydrochloride), a relatively selective inhibitor of DHCR7 (12–14). We previously described progressive retinal degeneration in this rat model of SLOS, which is characterized by the shortening of retinal rod outer segments, pyknosis and thinning of the outer nuclear layer (ONL) of the retina (which contains the photoreceptor nuclei), and accumulation of membranous/lipid inclusions in the retinal pigment epithelium (RPE) (12, 13). Reduced rod outer segment membrane fluidity, primarily caused by a dramatic (30 to 40 mol%) decline in docosahexaenoic acid (22:6, n3) levels relative to age-matched controls, also was observed in the SLOS rat model by three postnatal months (15, 16). Retinal function and sterol steady-state in the same rat model of SLOS can be partially rescued using a high-cholesterol diet (2% by weight), although histopathological degeneration of the retina still occurs (17). However, the molecular mechanisms that underlie the observed electrophysiological defects in the retina, the accumulation of membranous/lipid inclusions in the RPE, the shortening of retinal rod outer segments, and the initiation of ONL pyknosis in the SLOS rat model remain poorly understood. Therefore, a comprehensive profiling of the retinal proteomes of AY9944-treated versus age-matched untreated control rats may contribute to further understanding of the underlying mechanisms responsible for the retinopathy associated with the SLOS model and, by extension, the human disease.

Nevertheless, extensive and reliable expression profiling of the retinal proteome remains a prominent challenge, owing to the need to quantify data from multiple animals and a high percentage of integral membrane and membrane-associated proteins (18, 19). Label-free approaches can compare multiple replicates (20–22) with quantitative accuracy comparable to that attained with stable isotope-labeling methods (23–25). However, in order to achieve reliable relative quantification, highly quantitative and reproducible sample preparation and LC/MS analysis are required for relatively large-scale sample cohorts.

In the present study, we performed a reproducible, well-controlled, ion-current-based comparative proteomic analysis of the retinas from AY9944-treated versus age/sex-matched control rats (n = 5 animals per group). A high-concentration detergent mixture was used for the efficient extraction of proteins from retinas, and samples then underwent a reproducible precipitation/on-pellet-digestion procedure and long-column, 7-h nano-LC-MS analysis. These approaches ensured extensive comparative analysis of retina samples with 10 animals. The preparative and analytical procedures were carefully optimized and controlled to ensure optimal reproducibility. Two label-free approaches, the ion-current-based method and a spectral counting method, were compared in parallel. The altered proteins were subjected to functional annotation, and selected groups of proteins of interest were further validated by means of Western blot and correlative immunohistochemical analysis.

**EXPERIMENTAL PROCEDURES**

**Materials and Reagents**—Please see the supplemental “Experimental Procedures” section for details.

**Treatment of Animals**—The SLOS rat model was generated essentially as previously described by Fliesler et al. (12), with modifications as described by Fliesler et al. (17). For each treatment versus control comparison, all animals were age- and sex (male)-matched. All procedures involving animals were approved by the Buffalo Veterans Administration Medical Center (VAMC) and University at Buffalo Institutional Animal Care and Use Committees and were in accordance with the ARVO Resolution on the Use of Animals in Research and with the NIH Guide for the Care and Use of Laboratory Animals.

**Tissue Preparation and Precipitation/On-pellet Digestion**—Each isolated retina was homogenized in 400 μl of ice-cold lysis buffer (50 mM Tris-formic acid, 150 mM NaCl, 0.5% sodium deoxycholate, 2% SDS, 2% Nonidet P-40, pH 8.0) with a Polytron homogenizer (Kinematica AG, Lucerne, Switzerland). Homogenization was performed for 5 to 10 s at 15,000 rpm and was followed by a 20-s cooling period to allow the foam to settle; this procedure was repeated 10 times. The mixture was then sonicated with a low-power bath sonicator in a cold room (4 °C) for ~10 min, after which the solution was clear, and sonication was followed by centrifugation at 140,000 × g for 1 h at 4 °C. The supernatant was carefully transferred to a fresh tube, sealed, and stored at −80 °C until analysis. In order to remove undesirable components in the samples while maintaining high peptide recovery, we employed a precipitation/on-pellet-digestion protocol modified from that described previously (26); the details are shown in the supplemental “Experimental Procedures” section.

**Nano-reverse-phase Liquid Chromatography/Mass Spectrometry**—The nano-reverse-phase liquid chromatography system consisted of a Spark Endurance autosampler (Emmen, Holland) and an ultra-high-pressure Eksigent (Dublin, CA) Nano-2D Ultra capillary/nano-LC system. To achieve comprehensive separation of the complex peptide mixture, a nano-LC/nanospray setup that featured a low void volume and high chromatographic reproducibility (26) was employed. Mobile phases A and B were 0.1% formic acid in 2% acetonitrile and 0.1% formic acid in 88% acetonitrile, respectively. Samples (each containing 6 μg of total peptides) were loaded onto a large-inner-diameter trap (300 μm inner diameter × 1 cm, packed with Zorbax 3-μm C18 material) with 1% mobile phase B at a flow rate of 10 μl/min, and the trap was washed for 3 min. A series of nanoflow gradients (flow rate, 250 nl/min) was used to back-flush the trapped samples onto the nano-LC column (75 μm inner diameter × 75 cm, packed with Pepmap® 3-μm C18 material) for separation. Heating the nano-LC column to 52 °C greatly improved both chromatographic resolution and reproducibility. A 7-h gradient was used to resolve the complex peptide mixture, which consisted of the following steps: 3% B for 10 min, 3%–8% B in 5 min, 8%–24% B in 145 min, 38% B over 95 min, 38%–63% B over 55 min, 97% B in 35 min, isocratic condi-
tions at 97% B for 25 min, and finally 3% B for 40 min. In order to maintain stabilized ionization efficiency, the spray tip was washed by dripping 50% methanol by gravity after every three runs.

An LTQ/Orbitrap-ETD hybrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA) was used for protein identification. The instrument was operated in data-dependent product ion mode. One scan cycle included an MS1 scan (m/z 300–2000) at a resolution of 60,000 followed by seven MS2 scans in collision-induced dissociation activation mode to fragment the seven most abundant precursors found in the MS1 spectrum. The target value for MS1 by Orbitrap was 7 × 10^6, under which the Orbitrap was calibrated carefully for mass accuracy and Fourier transform transmission. The use of a high target value on the Orbitrap enabled highly sensitive detection without compromising the mass accuracy and resolution. The activation time was 30 ms, the isolation width was 1.5 Da for the linear ion trap (LTQ), the normalized activation energy was 35%, and the activation q was 0.25.

Five biological replicates of each biological group (AY9944-treated versus control) were analyzed randomly; in addition, 10 replicates of “Sham” samples (equal mixture of AY9944-treated and control material) were analyzed to assess the quantitative reproducibility and precision of the procedure. Therefore, in total, 20 nano-LC/MS runs of the biological and Sham groups were performed in a randomly interleaved sequence.

**Protein Identification**—Individual data files were searched against the Swiss-Prot protein database (June 13, 2012) with a total of 7,766 protein entries using Proteome Discoverer 1.2 (Thermo-Scientific). The search parameters used were as follows: 25-ppm tolerance for precursor ion masses and 1.0 Da for fragment ion masses. Two missed cleavages were permitted for fully tryptic peptides. Carbamidomethylation of cysteines was set as a fixed modification, and a variable modification of methionine oxidation was allowed. The false discovery rate was detected by using a target-decoy search strategy (27). The sequence database contains each sequence in both forward and reversed orientations, enabling false discovery rate estimation.

**Scaffold 3.6** (Proteome Software, Portland, OR), capable of handling large-scale proteomic datasets, was used to validate MS/MS-based peptide and protein identification based on cross-correlation (Xcorr) and Delta Cn values. The peptide filtering criteria included Delta Cn scores of >0.1 and Xcorr scores of >1.7, 1.9, 2.3 and 2.5, respectively, for singly, doubly, triply, and quadruply charged peptides. Stringent cutoffs for the Delta Cn scores and Xcorr values were used to filter the data. The peptide filtering criteria included Delta Cn scores of >0.1 and Xcorr scores of >1.7, 1.9, 2.3 and 2.5, respectively, for singly, doubly, triply, and quadruply charged peptides. Stringent cutoffs for the Delta Cn scores and Xcorr values were used to filter the data. The peptide filtering criteria included Delta Cn scores of >0.1 and Xcorr scores of >1.7, 1.9, 2.3 and 2.5, respectively, for singly, doubly, triply, and quadruply charged peptides. Stringent cutoffs for the Delta Cn scores and Xcorr values were used to filter the data. The peptide filtering criteria included Delta Cn scores of >0.1 and Xcorr scores of >1.7, 1.9, 2.3 and 2.5, respectively, for singly, doubly, triply, and quadruply charged peptides. Stringent cutoffs for the Delta Cn scores and Xcorr values were used to filter the data. The peptide filtering criteria included Delta Cn scores of >0.1 and Xcorr scores of >1.7, 1.9, 2.3 and 2.5, respectively, for singly, doubly, triply, and quadruply charged peptides. Stringent cutoffs for the Delta Cn scores and Xcorr values were used to filter the data. The peptide filtering criteria included Delta Cn scores of >0.1 and Xcorr scores of >1.7, 1.9, 2.3 and 2.5, respectively, for singly, doubly, triply, and quadruply charged peptides. Stringent cutoffs for the Delta Cn scores and Xcorr values were used to filter the data. The peptide filtering criteria included Delta Cn scores of >0.1 and Xcorr scores of >1.7, 1.9, 2.3 and 2.5, respectively, for singly, doubly, triply, and quadruply charged peptides. Stringent cutoffs for the Delta Cn scores and Xcorr values were used to filter the data. The peptide filtering criteria included Delta Cn scores of >0.1 and Xcorr scores of >1.7, 1.9, 2.3 and 2.5, respectively, for singly, doubly, triply, and quadruply charged peptides. Stringent cutoffs for the Delta Cn scores and Xcorr values were used to filter the data. The peptide filtering criteria included Delta Cn scores of >0.1 and Xcorr scores of >1.7, 1.9, 2.3 and 2.5, respectively, for singly, doubly, triply, and quadruply charged peptides. Stringent cutoffs for the Delta Cn scores and Xcorr values were used to filter the data. The peptide filtering criteria included Delta Cn scores of >0.1 and Xcorr scores of >1.7, 1.9, 2.3 and 2.5, respectively, for singly, doubly, triply, and quadruply charged peptides. Stringent cutoffs for the Delta Cn scores and Xcorr values were used to filter the data.

**Protein Quantitation and Evaluation**—Ion-current-based quantitative analysis was performed using SIEVE® v2.0 software (Thermo Scientific, San Jose, CA) to obtain area-under-the-curve (AUC) data, and then a sum-intensity method was used to aggregate the quantitative data from peptide level to protein level. SIEVE® is a label-free differential expression package that includes chromatographic alignment and global intensity-based MS1 feature extraction (29). The SIEVE® package processed chromatographic alignment between LC-MS/MS runs with the ChromAlign algorithm (30). In order to achieve excellent quality control of LC-MS/MS runs, the alignment scores and base-peak-ion-current intensity were closely monitored and benchmarked during sample runs. Subsequently, quantitative “frames” were defined based on m/z and retention time in the aligned collective data set. In order to ensure the reliability of quantification, only frames with high-quality AUC data (e.g. signal-to-noise ratio > 10) were used for quantification. Peptide ion current areas were calculated for each frame. Subsequent to relative quantification, we identified MS2 fragmentation scans associated with each frame by importing the msf files created by Proteome Discoverer (see description of the protein identification procedure). Peptides shared among different protein groups were excluded from quantitative analysis. The ion current intensities of each protein were normalized by the total AUC in each individual run. Statistical significance between groups (comparing treated versus control specimens) was evaluated using a Student’s t test, with a p value cutoff of 0.05. The relative expression ratio was calculated using the average ion current intensities of five replicates in each group.

To estimate quantitative variability arising from technical aspects, we analyzed 10 replicates of the same pool sample along with the AY9944-treated versus control data set (Fig. 1). The dataset was processed by randomly selecting five runs as the “Sham-control” group and the rest as the “Sham-experimental” group. Precision, accuracy, and false positives among the two groups were evaluated.

We compared the performance of spectral counting and ion-current-based methods. Details of the spectral counting are shown in the supplemental “Experimental Procedures” section.

**Western Blot Analyses**—For Western blot analyses, retina protein samples (30 µg each) in lysis buffer (50 mM Tris-formic acid, 150 mM NaCl, 0.5% sodium deoxycholate, 2% SDS, 2% Nonidet P-40, pH 8.0) were first separated via SDS-PAGE using small-format 10% or 4%/12% linear gradient polyacrylamide gels (Invitrogen) using standard methodology. Proteins then were electroforetically transferred to polyvinylidene difluoride membranes (Invitrogen). The membrane was blocked for 1 h with Western blocking solution (Invitrogen) and sequentially incubated with a primary antibody followed by an appropriate secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Inc., Dallas, TX). The positive immunoreactions were detected with x-ray film via chemiluminescence using an ECL Western blotting kit (Pierce, Rockford, IL) and developed with a Kodak X-Omat 2000A processor. When a Western blot was re-probed with a different primary/secondary antibody set, Restore PLUS® Western blot stripping buffer was employed, per the manufacturer’s directions (Thermo Fisher). The primary antibodies used in this study were as follows: rabbit polyclonal anti-sterol regulatory element-binding protein 1 (SREBP-1) (1:500; Santa Cruz Biotechnology), rabbit polyclonal anti-SREBP-2 (1:500; Santa Cruz Biotechnology), rabbit polyclonal anti-caspase-3 (1:250; Cell Signaling Technology, Danvers, MA), rabbit polyclonal anti-cleaved caspase-3 (1:250; Cell Signaling Technology), rabbit polyclonal anti-cathepsin D (1:500; Santa Cruz Biotechnology), mouse monoclonal anti-β-gal actin (1:2,000; Santa Cruz Biotechnology), mouse monoclonal anti-β-actin (1:2,000; Santa Cruz Biotechnology), and mouse monoclonal anti-β-actin (1:2,000; Santa Cruz Biotechnology). Proteins were electrophoretically transferred out essentially as described by Shi et al. (31). Slides were placed into 50-ml conical polypropylene tubes containing 10 mM sodium citrate, pH 6.1 (preheated in boiling water), and then heated in a boiling water bath for 20 min, after which they were cooled at ambient room temperature for 20 min. After brief re-equilibration in PBS, tissue sections were blocked for 1 h with 1% (w/v) “RIA grade” bovine serum albumin (BSA), 0.25% (v/v) Triton X-100, and 5% (v/v) normal donkey serum (all from Sigma-Aldrich, St. Louis, MO) in 20 mM Tris-buffered saline (TBS; 150 mM Tris, 0.9% (w/v) NaCl, pH 7.4). For primary antibody treatments, sections were incubated with immunoreagents.
diluted in BSA/Triton X-100/TBS (antibody diluent) in a humidified chamber. Primary antibodies employed were GFAP (1:100), anti-caspase-3 (full length and cleaved; 1:50 to 1:200), and anti-cathepsin D (1:200 to 1:500) as described above. Negative control incubations were with normal rabbit IgG (10 μg/ml; Vector Labs, Burlingame, CA) or without primary antibody (data not shown). Anti-cleaved caspase-3 antibodies were validated using positive control slides of etoposide-treated Jurkat cells (Cell Signaling Technology). After serial rinses with 0.025% Triton X-100/TBS followed by TBS alone, sections were treated with donkey anti-rabbit IgG antibody conjugated with Alexa Fluor 568 (Molecular Probes, Eugene, OR) at 5 μg/ml in antibody diluent for 45 min at room temperature. Slides were rinsed in TBS, coverslipped using Vectashield® containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Labs) to label cell nuclei, and stored protected from light at 4 °C. Sections were examined via bright-field and epifluorescence microscopy, and images were captured digitally using an Olympus BH-2 epifluorescence microscope (Center Valley, PA) equipped with a Nikon DXM1200 digital camera (Nikon Inc., Melville, NY) controlled by a Microsoft Windows™-based personal computer. Full-thickness, unprocessed images of retinas from both control and AY9944-treated animals were taken at equivalent excitation wavelength intensities and camera sensitivity settings (ACT-1 software, Nikon) using an Olympus DPlanApo 20X UV (0.70 NA) objective.

RESULTS
Development and Evaluation of the Ion-current-based Procedure for Profiling of Retinal Proteome—In order to achieve a reliable discovery of altered proteins in the AY9944-induced retinal degeneration in the SLOS rat model, we developed a reproducible, extensive, and well-controlled proteomic strategy (see Fig. 1) for profiling of the retinal proteome with 10 animals. All samples were prepared and analyzed in a random order to obviate analytical bias. Each of the following steps was thoroughly optimized and evaluated.

Bioinformatics Analysis—Protein function and pathway analyses were carried out using Ingenuity Pathway Analysis. Ingenuity’s knowledge base is created via manual curation of the scientific literature supported by experimental results that are structured into an ontological relational database. We analyzed the data set of 101 differentially expressed proteins determined via the ion-current-based method. The data set was imported directly into Ingenuity Pathway Analysis for a Core analysis. Biological functions assigned by the software were manually examined and re-grouped into respective categories. Hierarchical cluster analysis was performed using Cluster 3.0 (33) and displayed by TreeView (Java software), which supports tree-based and image-based browsing of hierarchical trees. In the heat map generated by TreeView, the elevated protein intensities are indicated in red, and the decreased protein intensities are indicated in green.
Efficient and Quantitative Retinal Protein Extraction, Sample Cleanup, and Digestion—The retina is a highly ordered tissue with various anatomical layers, cell types, and subcellular compartments containing numerous hydrophobic membrane proteins (34); thus, it is challenging to extract the membrane-bound proteins efficiently and quantitatively. To address this problem, we homogenized retinas using a Polytron tissue homogenizer in a “strong” buffer containing a mixture of detergents (2% Nonidet P-40, 2% SDS, and 0.5% sodium deoxycholate) that has been previously demonstrated by our lab to efficiently extract proteins from biological tissues, including membrane proteins (22, 26, 35, 36). The extracted proteins were subjected to a quantitative precipitation/on-pellet-digestion procedure (22, 26, 37) to remove detergents and other non-protein components and then completely digest the extracts. Under the optimized conditions, high, reproducible protein yields were achieved (supplemental Fig. S1A). The completeness of the extraction was confirmed by the observation that <0.5% of the total proteins were detected in the residue tissue pellets (data not shown). After digestion, the mean peptide recovery was 80.5% with a relative standard deviation of 11.9% ($\sigma = 10$, determined via a modified BCA method (26)), and day-to-day reproducibility was excellent as well (supplemental Fig. S1B). Among the proteins analyzed in this study, a total of 473 (40.5%) proteins out of the 1,167 proteins with available Gene Ontology Cellular Location information were assigned as membrane (i.e. highly hydrophobic) proteins, indicating the good recovery of membrane proteins.

Reproducible and Sensitive Nano-LC/MS Analysis with Extensive Chromatographic Separation—We developed and optimized a high-resolution and reproducible chromatographic strategy that permits extensive and reliable quantification of the retinal proteome with multiple biological replicates. The run-to-run reproducibility was assessed by performing 10 consecutive analyses of a pooled retina sample over a three-day period. Ten representative peptides that were randomly selected within each 30-min segment of the elution window were used for the evaluation. Low variations of 0.3% to 0.7% and 5.8% to 14.8% for the retention times and AUCs, respectively, across the 10 peptides were achieved. We also assessed the quantitative reproducibility on protein levels using both the ion-current-based and the spectral counting methods. Some representative data are shown in Fig. 2. The results not only demonstrated the high reproducibility of the technique developed, but also indicated that the ion-current-based approach provided superior quantitative precision. In comparison of the 1st analysis versus the 3rd, 6th and 10th analyses, both spectral counting and ion-current-based methods showed Pearson’s correlation coefficients ($\rho$)

![Fig. 2. Evaluation of the analytical accuracy and precision for quantification of the retina proteome by performing 10 analyses of a pooled sample. Some representative plots of linear correlations between runs are shown. A, representative plots for two analyses via spectral counting: the 3rd, 6th, and 10th runs versus the 1st run. B, representative plots for two analyses via the ion-current-based method: the 3rd, 6th, and 10th runs versus the 1st run. Pearson’s correlation coefficient ($\rho$) is shown for each comparison.](image-url)
58 elevated proteins
43 decreased proteins

Fig. 3. Hierarchical clustering analysis of the 101 differentially expressed proteins observed in retina from AY9944-treated rats (n = 5, AY9944_1 to AY9944_5) and controls (n = 5, Control_1 to Control_5). The elevated protein intensities are indicated in red, and the decreased protein intensities are indicated in green.

Significantly Altered Proteins in Retinas from SLOS Model Rats versus Age-matched Controls—As one of the purposes of this study was to discovery altered proteins with high confidence, a set of stringent cutoff criteria were applied so that only proteins/peptides with high-quality AUC data were quantified. These included high-cutoff criteria for peptide identification, strict criteria for peak detection and frame generation (e.g. signal-to-noise ratio > 10), and the requirement that each quantifiable protein have at least two independent sets of qualified AUC data from distinct peptides (see "Experimental Procedures"). Furthermore, peptides shared among different proteins were removed from quantitative analysis. Under these criteria, 1,259 unique protein groups were quantified in the AY9944-versus-control sample set, which encompasses the quantification of the ion currents from 9,817 unique peptides. There were no missing data in any replicate on the protein level in either the AY9944-versus-control sample set or the Sham sample set. In this study, the cutoff criteria of 30% change in either direction and p < 0.05 (AY9944-treated versus control group) were employed for differential expression analyses based on the estimation of false positives (shown above). As a result, 101 proteins were determined as altered. The mean value of sequence coverage of 1,259 proteins analyzed in this study was 27.9% (supplemental Table S3). The biological variation among retinas from AY9944-treated versus control rats (n = 5 each) was evaluated as well. The mean coefficient of variation values of ion current areas for all quantified proteins were 16.0% and 14.0%, respectively, in the five AY9944-treated animals and five controls. Of the 101 differentially expressed proteins determined via the ion-current-based approach (supplemental Table S4), 58 were elevated and 43 were decreased in retinas from the AY9944-treated group relative to the age-matched control group. Fig. 3 shows a heat map plot of the intensities of altered proteins in the 10 biological samples and illustrates the significant change of the 101 proteins between AY9944-treated and control animals. As indicated in the “tree” diagrams, five AY9944-treated samples and five controls were...
classified into the corresponding clusters as expected. These changes in protein abundance are also reflected in the volcano plots (fold change versus p values) of the AY9944/control set (supplemental Fig. S3). By comparison, in the Sham set, the vast majority of proteins showed a ratio close to 1, with no altered proteins discovered, which further illustrates the high accuracy and reproducibility of the overall proteomics strategy. The ratios of peptides from altered proteins were further investigated (see supplemental Table S5). The distribution of peptide ratios assigned to altered proteins is shown in supplemental Fig. S4. The majority of the 368 unique peptides assigned to the 58 elevated proteins (351, 95.4%) had an elevated expression ratio (supplemental Fig. S4A). As a randomly selected example, GFAP, one of the more profoundly altered proteins, was elevated and quantified with 26 distinct peptides; of these peptides, 25 (96.2%) had relative ratios greater than 1.3 (supplemental Table S5). A similar trend was observed for the peptides in decreased proteins (supplemental Fig. S4B). This result demonstrates that corresponding peptide ratios agree well with the protein ratios achieved in this study.

Moreover, we compared the relative expression ratios of these 101 proteins obtained via spectral counting (supplemental Table S6) and ion-current-based approaches. Interestingly, the ratios obtained via the two methods showed strongly significant correlation for the altered proteins (Pearson's correlation coefficient = 0.81, p < 0.001), as specified in supplemental Fig. S5. Furthermore, the levels of "housekeeping" proteins, such as β-tubulin, actin, and glyceraldehyde-3-phosphate dehydrogenase, which are often used as the loading controls for SDS-PAGE and Western blot analysis, remained unchanged between the two groups (supplemental Fig. S6) as determined via both the ion-current-based and the spectral counting methods.

**Functional Annotation and Validation of Proteins of High Interest**—The 101 altered proteins were assigned to 10 major categories of biological processes using Ingenuity Pathway Analysis and manual literature investigation. The 10 categories reflect key biological processes perturbed in the retinal degeneration associated with the SLOS model, which are shown in Fig. 4 and supplemental Table S4. The altered proteins in categories such as lipid metabolism, cell death, oxidative stress response, visual perception, and vesicle-mediated transport were consistent with previously published histological and metabolic observations regarding retinal degeneration in the SLOS rat model (12, 16). Among the differentially altered proteins discovered via the ion-current-based method, five proteins of high interest—ApoE, cathepsin D, GFAP, Stat3, and μ-crystallin—were validated by Western blot analysis. The rationale for the selection of these particular proteins is provided below. We further evaluated the differential expression of the cholesterol/lipid regulatory proteins SREBP-1 and SREBP-2 and the pro-apoptotic protein caspase-3. Moreover, cathepsin D, GFAP, and caspase-3 were validated by means of correlative immunohistochemistry using retinal tissue sections and monospecific antibodies against these proteins. A TUNEL assay also was performed to investigate cell death in AY9944-treated versus control retinas.

**Concordance of Ion-current-based Proteomic Data and Western Blot Results**—Of the five selected proteins of interest, cathepsin D (CATD), GFAP, Stat3, and μ-crystallin were elevated and ApoE was decreased in the AY9944-treated group relative to controls; in contrast, levels of β-actin (a housekeeping gene product) were not different in the two groups (see Fig. 5A). The mean intensities in control retinas were normalized to 1.0 for both Western blot assay and ion-current-based quantification and were found to be consistent with one another (Fig. 5B).

**Oxidative Stress Response**—We found nine significantly altered proteins relevant to oxidative stress responses (supplemental Table S4). The expression levels of ApoE and μ-crystallin were further validated by Western blot analyses, as shown in Fig. 5.

**Proteins Associated with Lipid Metabolism**—We found that 19 of the differentially expressed proteins were relevant to lipid metabolism (see Fig. 4), which is consistent with the known biochemical hallmarks of both the human disease and the SLOS rat model (16). Given that the SLOS-associated defect is in cholesterol biosynthesis, we hypothesized that the expression/regulation of SREBPs would be altered in the SLOS model. As shown by Western blot analysis (Fig. 6), significant increases in the levels of both precursor SREBP-1 (M, ~125 kDa) and its mature, cleaved form (cSREBP-1, M, ~68 kDa) were observed in retinas of AY9944-treated rats (at approximately 2 months postnatal age) relative to untreated, age-matched controls. However, AY9944 treatment de-
increased the level of cleaved SREBP-2 (cSREBP-2) (Mr, ~68 kDa; Fig. 6). Among the 19 altered proteins associated with lipid metabolism, apolipoprotein A1 (ApoA1) (decreased 5.1-fold), ApoE (decreased 1.7-fold), \(\alpha\)-2-macroglobulin (elevated 3.7-fold), haptoglobin (elevated 3.4-fold), and paraoxonase-1 (decreased 1.4-fold) are known to be involved in cholesterol metabolism or transport. The changes in ApoE levels were further validated by Western blot analysis, as shown in Fig. 5B. These findings are considered within the context of global alterations in lipid metabolism (see "Discussion").

Caspase-3-independent Cell Death Pathway in AY9944-treated Retinas—Extensive TUNEL-positive labeling was observed in the ONL of retinas from AY9944-treated rats (Fig. 7B); by contrast, untreated control retinas exhibited only occasional TUNEL-positive cells (Fig. 7A). As the ONL normally contains the nuclei of only the rod and cone photoreceptor cells, these findings are consistent with the observed retinal degeneration in the SLOS rat model (12, 38). Notably, TUNEL-positive labeling was restricted almost exclusively to the ONL, suggesting that photoreceptor cells are somehow selectively (or more highly) sensitive to the associated cytotoxic effects of AY9944 treatment. In addition, GFAP, a sensitive marker for the activation and hypertrophy of glia (a.k.a. gliosis), as occurs in various types of retinal degeneration (39, 40), was found to be highly expressed in retinas of AY9944-treated rats via ion-current-based profiling (Fig. 3). The elevated (relative to controls) expression of GFAP was further confirmed by both Western blot and correlative immunohistochemical analyses, as shown in Fig. 8. The radial labeling pattern (Fig. 8C, green immunofluorescence) extending from the inner limiting membrane through the ganglion cell layer, the inner plexiform layer, and the outer retina is consistent with activated Müller glia. By contrast, retinas from control animals (Fig. 8B) were quiescent, exhibiting only the expected sparse anti-GFAP immunoreactivity along the inner limiting membrane (the location of astrocytes and the "end feet" of the Müller glia).

In this study, 42 significantly altered proteins in the AY9944-treated group, relative to age-matched controls, were associated with cell death, as shown in Fig. 4. Among these, proteins highly expressed (>2-fold) in retinas of AY9944-treated rats, such as cathepsin D, clusterin, HSPB1, and
Stat3, have been reported previously to be involved in caspase-3-independent cell death pathways (see “Discussion”). Here, increased expression of cathepsin D and Stat3 was further validated by Western blot analyses and immunohistochemistry (for cathepsin D), as shown in Fig. 9 and supplemental Fig. S7, respectively. Pro-cathepsin D ($M_r \sim 52$ kDa) undergoes N-glycosylation and yields an intermediate form ($M_r \sim 48$ kDa) followed by the formation of a two-chain mature enzyme ($M_r \sim 34$ kDa and $\sim 14$ kDa) (41, 42). In this study, we found that the levels of pro-cathepsin D, the intermediate form, and the mature enzyme were substantially higher in retinas of AY9944-treated rats than in those of controls, as shown in Fig. 9A. Furthermore, correlative immunohistochemistry (Figs. 9B and 9C) demonstrated a dramatic increase of cathepsin D immunoreactivity, particularly in the RPE and ganglion cell layers, with only relatively low to moderate increased labeling in other layers of the neural retina, in AY9944-treated rats relative to controls (see “Discussion”).

We further evaluated the involvement of caspase-3 activation in the observed retinal degeneration, which involves cleavage of procaspase-3 by initiator caspases to generate an active heterotetramer (43). Fig. 10A shows that the levels of procaspase-3 ($M_r \sim 35$ kDa) were comparable in retinas from control and AY9944-treated rats, but there was no detectable cleaved caspase-3 ($M_r \sim 19$ kDa) in retinas under either condition. By contrast, cytochrome c-treated Jurkat cell extracts (a positive control with low levels of cleaved caspase-3 (44)) exhibited the expected 19-kDa cleavage product, whereas untreated Jurkat cell extracts (the negative control) did not show this cleavage product, which validated the activity and sensitivity of the anti-cleaved-caspase antibody used in this study. Correlative immunohistochemistry, using the same anti-cleaved-caspase antibody on paraffin sections of retinas from AY9944-treated and control rats, did not show cleaved caspase-3 ($M_r \sim 19$ kDa) in retinas under either condition. By comparison, the parallel treatment of paraffin sections of intact Jurkat cells pretreated with etoposide (a pro-apoptotic agent) showed predominantly immunopositive cells (data not shown), demonstrating that fixation and paraffin embedding of cells do not diminish the activity of the antibody (i.e. if cleaved caspase-3 had been present in the retina specimens, we would have been able to detect it via this immunohistochemical method). Un-treated fixed/paraffin-embedded Jurkat cells were not immunopositive (negative control). Thus, these results strongly support
the significant and novel finding that cell death in the retina of the SLOS rat model is caspase-3-independent.

**DISCUSSION**

Decreased levels of cholesterol and elevated levels of 7DHC typically are observed in SLOS patients (1, 2). Fliesler et al. previously described a rat model of SLOS created via the treatment of pregnant female rats with AY9944 during the second and third gestational weeks followed by the systemic injection of the progeny with the drug for up to three postnatal months (12, 13, 17). In this model, gross elevation of 7DHC levels and reduction of cholesterol levels are detected in the blood, retina, liver, and brain, and the extent of these biochemical alterations tends to be even greater than what is observed typically in SLOS patients. In the present study, to provide insight into the molecular mechanisms underlying the observed retinal degeneration in the SLOS rat model and, by inference, the human disease, we performed proteomic profiling of retinas of AY9944-treated rats in comparison with those from age-matched control rats. The following sections discuss both the well-controlled proteomics strategy and key discoveries derived therefrom.

**Well-controlled, Extensive, and Accurate Ion-current-based Profiling of the Retinal Proteomes in 10 Animals**—In order to achieve extensive and confident discovery of the regulatory proteins that are potentially responsible for retinal degeneration in the SLOS model, it is critical to develop extensive, quantitative, and reproducible procedures for sample preparation (especially for the membrane proteins in various subcellular compartments of the retina), analysis, and quantification and to minimize the extent of false positives in discovering altered proteins.

In the present study we optimized a high-resolution and reproducible chromatographic strategy that permitted extensive and reliable quantification of the retinal proteome with 10 animals. An Orbitrap analyzer with an “overfilling” approach to enhance sensitivity without compromising MS accuracy and resolution (26, 35, 36) was used to produce the precursor ion currents for quantification. Using a low-void-volume and reproducible nano-LC/nanospray configuration modified from what we had developed previously (22, 26, 35, 37), we extensively resolved retinal samples on a long, heated reverse-phase nano-LC column (75 cm long with 3-μm particles and heated at 52 °C) with a shallow, 7-h gradient. A wide peptide elution window (315 min) with an average peak width of less than 30 s (full width at half-maximum) and a peak capacity greater than 600 was achieved, which enabled in-depth profiling of the retinal proteome. The homogeneous heating of the column and the use of a large-inner-diameter trap promoted efficient gradient mixing and dampened pump noise, allowing highly reproducible separation. Furthermore, regular rinsing of the spray tip (see “Experimental Procedures”) considerably improved the reproducibility of the MS signal response. The level of reproducibility attained was a key factor in this study and permitted reliable quantification using tissues obtained from 10 animals without the need for isotopic labeling. Evaluation data demonstrated the high quantitative precision and accuracy of the developed method (see Fig. 2 and
supplemental Figs. S1–S4). Moreover, compared with the spectral counting method, the ion-current-based approach was observed to provide markedly less quantitative variation, higher accuracy (which is particularly sound for low-abundance proteins), and reduced false discoveries for profiling of the rat retina proteome. These results justified the use of the ion-current-based method in this study. The reliability of proteomic quantification was further enhanced by the adoption of stringent criteria for identification and quantification, and by the fact that each quantifiable protein had independent, high-quality AUC data (e.g. signal-to-noise ratio ≥ 10) from at least two distinct peptides.

**Altered Lipid Metabolism in Retinas of AY9944-treated Rats**—Our proteomics data are fully consistent with alterations in lipid metabolism that are not merely restricted to cholesterol biosynthesis. Nineteen altered proteins were associated with lipid metabolism. SREBP-1c, and SREBP-2), arising from two chromosomal loci. SREBP-1a and SREBP-1c preferentially regulate fatty acid and triglyceride metabolism, whereas SREBP-2 preferentially regulates the expression of genes involved in sterol metabolism pathways (46). However, SREBP-1c also can affect sterol synthesis rates when expressed at elevated levels. It was observed that the mature forms of SREBP-1 and SREBP-2 were increased in CaCo-2 cells depleted of cholesterol (48). Also, in the Dhcr7 knockout mouse model of SLOS (49), mature SREBP-2 was found to be increased significantly, apparently in response to the markedly decreased levels of cholesterol (50). Moreover, in a neuroblastoma cell line, shRNA-induced Dhcr7 knock-down resulted in decreased expression of the SREBP-2 gene relative to controls (51). Therefore, SREBP expression can be altered in different ways in response to cholesterol deficiency, depending on the experimental model and the method employed to cause deficiency. However, there are no reports documenting the protein expression profile of SREBPs in the AY9944-induced rat model of SLOS. Here, by means of Western blot analyses, we demonstrated elevated levels of mature (cSREBP-1) and precursor forms of SREBP-1 and decreased levels of mature SREBP-2 (cSREBP-2) in retinas of AY9944-treated rats relative to age-matched controls (Fig. 6). These findings are consistent with a regulatory feedback mechanism by which the synthesis of cholesterol is decreased in order to reduce the levels of potentially cytotoxic molecules (e.g. 7DHC-derived oxysterols) (52), which recently have been shown to form and accumulate in tissues of the AY9944 SLOS rat model (53, 54). Reduction of de novo biosynthesis of cholesterol in the retina of the SLOS rat model is suggested by the decreased cSREBP-2 levels. In addition, these findings would lead one to predict that the metabolism of fatty acids and sphingolipids, in addition to sterols, may be affected in retinas of AY9944-treated rats. However, studies aimed at directly evaluating the rates of biosynthesis and turnover of fatty acids and sphingolipids in the retina in the SLOS rat model have not been reported, and we prefer to refrain from further speculation on this topic until such direct evidence becomes available.

Mammalian cells obtain cholesterol via two pathways: (i) endogenous de novo synthesis in the endoplasmic reticulum, and (ii) receptor-mediated uptake of exogenous, cholesterol-rich low-density lipoproteins (55, 56). Though the mammalian retina has the capacity to synthesize its own cholesterol de novo (57, 58), the steady-state levels of cholesterol in the retina, as well in other tissues, are dramatically lower in the AY9944-treated rat model than in age-matched control rats (12, 13). In this study, our proteomic results suggest that the retina can respond to diminished cholesterol levels and perturbations in cholesterol biosynthesis by down-regulating reverse cholesterol transport (e.g. to retain sterols within the retina) and modulating other cholesterol metabolism and transport proteins (59, 60). For example, the increased ex-
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Expression of α-2-macroglobulin and haptoglobin and decreased expression of paraoxonase-1, ApoA1, and ApoE, as observed in the present study, potentially may reflect the retina’s homeostatic attempt to recover normal cholesterol levels. ApoA1 and ApoE, the major protein components of high-density lipoprotein, play an important role in the efflux of cellular cholesterol and phospholipids (61, 62). Paraoxonase-1 also increases the high-density-lipoprotein-mediated efflux of cholesterol, as previously reported (63). Lecithin-cholesterol acyltransferase, which converts free cholesterol to cholesterol esters with fatty acid from the sn-2 position of phosphatidylcholine, is of critical importance in reverse cholesterol transport (64). ApoA1 and ApoE are also the major activators of lecithin-cholesterol acyltransferase on apolipoprotein-B-containing lipoproteins (65, 66). Moreover, the enzymatic activity of lecithin-cholesterol acyltransferase can be inhibited by its binding to α-2-macroglobulin (67). In addition, it has been demonstrated that haptoglobin inhibits the reverse transport of cholesterol by preventing ApoA1 stimulation of lecithin-cholesterol acyltransferase activity (68). In sum, these observations suggest that, in response to cholesterol deficiency, retinal cells in AY9944-treated rats strive to recover normative cholesterol levels while also reducing the levels of potentially toxic sterol precursors (or toxic compounds subsequently derived from those precursors) via the suppression of sterol biosynthesis.

Cell Death Pathway and Retinal Degeneration in SLOS—Retinal degeneration, both inherited and induced, is characterized by a loss of vision due to the death of photoreceptors. Programmed cell death (or apoptosis) is a common pathway in most, if not all, known examples of photoreceptor loss both in humans and in animal models (69). The initial molecular basis of retinal degeneration in the SLOS rat model employed in this study is clear, as are the attendant histopathological and electrophysiological defects, which are comparable to or worse than those in the human disease (12). In this study we further confirmed photoreceptor cell death in this model, as evidenced by the massive extent of TUNEL-positive labeling in the ONL (Fig. 7). In addition, a marked increase of GFAP, a sensitive marker for gliotic reactivity in retinal degeneration, also was detected with proteomic profiling and validated by means of immunohistochemistry (Fig. 8). Despite these observations, the specific kind of cell death (e.g. apoptosis, necrosis, necroptosis, etc.) involved and a detailed accounting of molecular changes beyond those involved in cholesterol biosynthesis remained elusive. Both caspase-dependent and caspase-independent pathways have been implicated in retinal cell death, and there is still debate regarding which pathways may be involved, depending on the root cause (e.g. genetic, environmental, trauma) of the degeneration (69–71). For example, activation of caspase-3 has been reported in the rd-1 mouse model of retinal degeneration and in photoreceptor degeneration due to exposure to intense blue light (72, 73). However, Zeiss et al. demonstrated that caspase-3 did not appear to regulate rod cell death in an rd-1 mouse line in which caspase-3 had been concomitantly knocked out (74). Caspase-independent photoreceptor cell death also has been described in retinal degeneration induced by exposure to intense constant light (i.e. the “retinal light-damage” paradigm), and further studies show that this light-driven paradigm activates a caspase-independent pathway involving cathepsin D (70, 75). In addition, studies employing z-VAD-fmk, a broad-spectrum caspase inhibitor, failed to demonstrate protection of photoreceptor cells from death in the retinal light-damage model (44).

In the present study, we observed highly elevated levels of cathepsin D, clusterin, HSPB1, and Stat3, which collectively are consistent with caspase-3-independent cell death in retinas in the SLOS rat model. Cathepsin D has been implicated in a caspase-independent cell death pathway in light-induced retinal degeneration models (75); moreover, clusterin was shown to contribute to caspase-3-independent neuronal death (76). Stat3 has been reported to regulate a lysosomal-mediated pathway of cell death under physiological conditions independent of caspase-3 (77, 78). HSPB1 was observed to delay the release of cytochrome c from mitochondria and thus inhibit the activation of caspase-3 (79). Therefore, the elevation of these proteins suggests a caspase-3-independent cell death pathway in retinas from AY9944-treated rats. To validate this hypothesis (which was based upon proteomic data), we investigated the activation (cleavage) of caspase-3 using Western blot and correlative immunohistochemical analysis with an antibody immunospecific for cleaved caspase-3 that also has been validated as reactive in multiple species, including rat. As shown in Fig. 10, the presence of cleaved caspase-3 was not detected in retinas from either AY9944-treated rats or age-matched controls, yet the same antibody clearly recognized cleaved caspase-3 in positive controls (i.e. Western blot analysis of cytochrome c–treated cells and immunohistochemistry of etoposide-treated, paraffin-embedded cells). In sum, these results suggest that classical caspase-3-dependent apoptosis is not activated in the retinal degeneration associated with this rat model of SLOS.

In addition to caspases, the cathepsins, a family of cysteine, aspartate, and serine proteases, have been implicated in the regulation of programmed cell death (41, 80). Proteomics profiling detected significantly elevated levels of cathepsin D in retinas of AY9944-treated rats, which was further validated by Western blot and correlative immunohistochemistry. Levels of pro-cathepsin D isoforms, the intermediate form, and the mature enzyme were all notably higher in retinas of AY9944-treated rats than in controls, as shown in Fig. 9A. Cathepsin D also has been reported to cleave and activate Bax, resulting in the selective release of apoptosis-inducing factor from mitochondria and the induction of caspase-independent apoptosis (81). Therefore, by regulating apoptosis, the elevated cathepsin D levels in retinas of AY9944–treated
rads may play a role in SLOS model retinal degeneration. However, as shown in Fig. 9C, although cathepsin D immunoreactivity was moderately higher throughout the neural retina, RPE, and ganglion cells, the intensity was not higher in photoreceptors. As noted above, TUNEL-positive cells (Fig. 7) were found almost exclusively in the ONL, whereas there was no TUNEL labeling observed in the RPE or ganglion cell layers, indicating that those two cell types do not undergo appreciable cell death in this animal model (at least within the time window evaluated). Thus, the increase of cathepsin D in RPE and ganglion cells may be involved in cytoprotection, rather than in promoting cell death in this rat model of SLOS. In this regard, autophagy may be involved, affording these cell types a means to degrade damaged intracellular organelles and make new ones, rather than go down the path of cellular dysfunction, degeneration, and eventual death (82, 83).

Moreover, Stat3 was found to be elevated in retinas of AY9944-treated rats relative to controls (supplemental Fig. S7). Stat3 is a member of a family of cytokine-activated signaling molecules that can directly bind to DNA and modulate the transcription of target genes. The aberrant expression of Stat3 has been implicated in the regulation of cell proliferation, differentiation, transformation, and apoptosis (84). Furthermore, constitutive activation of Stat3 has been detected in various cancers and also has been shown to attenuate caspase-3-dependent apoptosis (85, 86). In addition, it has been demonstrated that Stat3 up-regulates the expression of cathepsins B and L and controls lysosomal-mediated cell death independent of executioner caspases-3, -6, and -7 (77). Therefore, we initially speculated that elevated expression of Stat3 in the retinas of AY9944-treated rats conceivably could play a regulatory role in determining the mode of cell death in the SLOS model retinal degeneration, driving the process toward a caspase-3-independent pathway, as suggested by the results discussed above. Furthermore, whereas cathepsin L was not quantified, cathepsin B was significantly elevated in retinas of AY9944-treated rats (AY9944-treated/control = 1.5-fold, p < 0.01). This observation was also consistent with a caspase-3-independent cell death mechanism in the SLOS rat model.

Visual System Development and Function—Previous studies showed that progressive retinal degeneration in the AY9944-treated rat model of SLOS was characterized by shortening of photoreceptor outer segments, pyknosis and thinning of the ONL, and electrophysiological abnormalities involving both rod and cone photoreceptors (12, 13). In the present study, we observed altered expression levels of nine proteins related to visual system development and function in the retinas of AY9944-treated rats relative to age-matched controls (supplemental Table S4). Among these, the level of rhodopsin kinase (GRK1) was reduced by 1.3-fold; GRK1 regulates the phototransduction cascade in the rod outer segments by phosphorylating the light-activated visual pigment rhodopsin, which in turn promotes the binding of phos-

rhodopsin to arrestin and prevents its further interaction with its cognate G-protein, transducin (Gt), thereby shutting off the visual transduction cascade (87). The guanine nucleotide binding protein α-transducin 3 (also known as gusducin) also was observed to be decreased by nearly 2-fold; this protein, originally associated with transduction mechanisms involved in the sensation of taste (88), more recently has been found to exist in mammalian retinas (89). The level of phosducin, which regulates the phototransduction cascade by interacting with transducin (90), also was found to be reduced by 1.6-fold. Peripherin-2 (also known as rd3), which plays a key role in the formation of photoreceptor outer segment disks and mutations that are responsible for various retinal degenerative diseases (91), also was decreased (1.5-fold) in retinas from AY9944-treated rats. In sum, the decreased levels of phototransduction-associated proteins found in this study are consistent with previous studies that have documented the visual dysfunction and concomitant retinal degeneration in the SLOS rat model.

In conclusion, we developed and then applied a reproducible and well-controlled ion-current-based strategy for the comparison of retinal proteomes from AY9944-treated rats versus age-matched controls. We discovered myriad changes in the expression levels of an expansive and diverse set of cellular proteins in the SLOS rat model of retinal degeneration, with high confidence. These molecular changes are predicted to have a significant effect on multiple biological processes and physiological functions, including lipid metabolism, oxidative stress response, cell death, proteolysis, visual transduction, and transport, consistent with the retinal degeneration observed in this animal model. Thus, to the extent that this pharmacologically induced rat model faithfully represents the underlying mechanism of the human genetic disorder, the pathobiology of SLOS goes well beyond the initial defect in cholesterol biosynthesis per se, as previously suggested (38). Moreover and beyond this, the proteomic strategies employed in this study represent technical advancements that may be applied more broadly to studying differences in protein expression in a wide variety of biological systems.

* This work was supported in part by U.S.P.H.S. (NIH) grants EY007361 (S.J.F.), U54HD071594 (J.Q.), DA027528 (J.Q.), and HL103411 (J.Q.); by a Center of Protein Therapeutics Industrial Award (J.Q.); by American Heart Association (AHA) award 12SDG9450036 (J.Q.); by an Unrestricted Grant from Research to Prevent Blindness (S.J.F.); and by facilities and resources provided by the Veterans Administration Western New York Healthcare System (S.J.F.).

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