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Spectrum and Range of Oxidative Stress Responses of Human Lens Epithelial Cells to H₂O₂ Insult

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Abstract

Purpose—Oxidative stress (OS) is believed to be a major contributor to age-related cataract and other age-related diseases.

Methods—cDNA microarrays were used to identify the spectrum and range of genes with transcript levels that are altered in response to acute H_2O_2 -induced OS in human lens epithelial (HLE) cells. HLE cells were treated with 50 μ M H_2O_2 for 1 hour in the absence of serum, followed by a return to complete medium. RNAs were prepared from treated and untreated cells at 0, 1, 2, and 8 hours after H_2O_2 treatment.

Results—The data showed 1171 genes that were significantly up- and downregulated in response to H_2O_2 treatment. Several functional subcategories of genes were identified, including those encoding DNA repair proteins, antioxidant defense enzymes, molecular chaperones, protein biosynthesis enzymes, and trafficking and degradation proteins. Differential expression of selected genes was confirmed at the level of RNA and/or protein. Many of the identified genes (e.g., glutathione *S*-transferase [MGST2], thioredoxin reductase β , and peroxiredoxin 2) have been identified as participants in OS responses in the lens and other systems. Some genes induced by OS in the current study (e.g., oxygen regulated protein [ORP150] and heat shock protein [HSP40]) are better known to respond to other forms of stress. Two genes (receptor tyrosine kinase [AXL/ARK] and protein phosphatase 2A) are known to be differentially expressed in cataract. Most of the genes point to a novel pathways associated with OS.

Conclusions—The present data provide a global perspective on those genes that respond to acute OS, point to novel genes and pathways associated with OS, and set the groundwork for understanding the functions of OS-related genes in lens protection and disease.

Oxidative stress (OS) occurs when the level of pro-oxidants (reactive oxygen species and other free radicals) exceeds the ability of the cell to respond through antioxidant defense and ultimately leads to modification and degradation of protein, damage to DNA and mitochondria, and cell death.^{1,2} Significant evidence points to oxidative damage as a major factor in the initiation and progression of numerous age-related diseases, including Alzheimer's and

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Parkinson's diseases, age-related macular degeneration, and age-related cataract.³ The transparent ocular lens is especially prone to oxidative damage, because the fiber cells of the lens are not renewed and therefore have to last a lifetime. Damage to these cells results in degradation of protein and ultimately in age-related cataract, which is the leading cause of blindness worldwide and the leading expense in Medicare-funded surgery in the United States. 3,4

To combat damage by OS and other insults, the numerous protective systems have evolved in the ocular lens, making it an excellent model to study both the biology of aging and the molecular mechanisms associated with OS.⁴ The lens is an avascular and encapsulated tissue comprising a single layer of epithelial cells that terminally differentiate into fiber cells. Fiber cell differentiation is characterized by elongation of the cells, synthesis and accumulation of crystallins, and eventual degradation of the nuclei and organelles. Thus, the main metabolic part of the lens and the only part of the lens capable of responding to environmental insults through altered gene expression is the lens epithelium.⁴ The lens epithelium is also the first part of the lens exposed to insults, including H_2O_2 . H_2O_2 is formed in the aqueous humor by a series of reactions triggered by the interaction of UV light with ascorbate and other molecules. Organ culture experiments have shown that acute OS induced by H_2O_2 treatment can irreversibly damage the lens epithelium, resulting in cell death and cataract.⁴

Among the systems that defend against OS in the lens are a high level of reduced glutathione (GSH),⁵ abundant antioxidant enzymes,⁶ and the chaperone-like functions of crystallins.⁷ Aging of the lens is characterized by a diminishing level of GSH and reduced activities of detoxifying enzymes.^{5,6} Numerous altered levels and activities of antioxidant enzymes and protective proteins have been detected after oxidative damage of lens epithelial cells.^{5,6,8} The lens also expresses a set of common transcription factors (e.g., AP-1, NF- κ B, p53, and upstream stimulatory factor [USF]), with activities that are regulated by the redox state of the cell,^{1,9} and these factors are known to activate batteries of genes that participate in protection and repair. Previous work has provided evidence that the human lens epithelium (HLE) is capable of responding to the presence of OS and cataract through the altered expression of numerous genes, including the regulatory subunit of protein phosphatase 2A,⁸ metallothionein IIa,¹⁰ thioltransferase,¹¹ catalase,¹² glutathione peroxidase,¹³ multiple glutathione *S*-transferases, ⁶ Na,K-ATPase,¹⁴ AP-1,¹⁵ and proline isomerase.¹⁶

These data suggest that the lens is capable of dynamic responses to OS and cataract, and in the present study we sought to elucidate the global cellular response of lens epithelial cells to H_2O_2 -induced stress by using high-throughput cDNA microarray technology and genome-scale analysis of the data.¹⁷ A consortium including Duke University (Durham, NC), the Fred Hutchinson Cancer Center (Seattle, WA), the Massachusetts Institute of Technology (Cambridge, MA), Oregon Health and Science University (Portland, OR), the University of Northern Carolina (Chapel Hill, NC), and the National Institute of Environmental Health Science (NIEHS, Bethesda, MD) is performing similar studies of OS response in human cells, including epithelial cells, fibroblasts, and lymphoblasts.¹⁸ The acute responses of lens cells with negligible apoptosis and necrosis. The present data establish six major functional gene classes and 96 specific genes that respond to acute nonlethal H₂O₂ treatment of HLE cells. The present results provide insight into those stress regulatory mechanisms that provide lens protection against OS, and the identified genes are candidates for the study of cataract and other stress-related disorders.

Methods

Cell Culture and RNA Preparation

HLE cells SR-01-04 were grown to 70% to 80% confluence in Dulbecco's modified minimum Eagle's medium in the presence of 15% fetal bovine serum, and gentamicin. The cells were treated with 50 μ M H₂O₂ for 1 hour in serum-free medium and then returned to complete medium and cultured for 2 and 8 hours (see Fig. 3). The control experiment was performed without H₂O₂. The cells were also treated similarly with H₂O₂ but grown for an additional 1 hour in the absence of serum. RNAs were prepared from separate experiments using a kit (Totally RNA; Ambion, Woodlands, TX).

Microarray Procedures

RNAs were analyzed by cDNA microarray hybridizations on human gene chips containing 9700 genes (developed at Albert Einstein College of Medicine [AECOM]).¹⁷ Reference RNA was prepared by pooling three RNA samples (see Fig. 4). Each hybridization was conducted in triplicate (i.e., technical replicates, because a single treatment of each cell culture did not yielded enough RNA for a biological replicate).¹⁹ Reference scanning, digitalization, normalization, and flagging of data were conducted (scanner and Genepix 3.02 software; Axon Instruments, Union City, CA, and GeneSpring 4.1 software; Silicon Genetics, San Carlos, CA). Normalized data tables can be obtained at http://www.aecom.yu.edu/thecvekllab.

Quality Control, Data Analysis, and Statistics

The entire raw data set was filtered to accommodate a requirement of at least two of three goodquality measurements for each triplicate experiment (the spot's average pixel intensity in both red and green channels must be greater than the average background pixel intensity plus 2 standard deviations). Normalization of intensity ratios across all experiments was performed based on medians of the 50th percentile. Log2-transformed ratios were further processed by the significance analysis of microarray (SAM) algorithm to generate a list of genes showing statistically significant up- or downregulation of transcripts.²⁰ We used stringent criteria for selection, to look for a set of genes with 1% probability of false discovery rate. Gene clustering, using self-organizing maps, was conducted with Genecluster (http://www.genome.wi.mit.edu; provided in the public domain by the Whitehead Institute, Massachusetts Institute of Technology, Cambridge, MA). Patterns of gene expression that formed subcategories specific to function were analyzed with the MultiExperiment Viewer (http://www.tigr.org; provided in the public domain by The Institute for Genomic Research [TIGR], Rockville, MD).

Semiquantitative RT-PCR

Primers were designed on computer (Prime algorithm from the GCG package; Oxford Molecular Group, Campbell, CA). The primer sets and reaction conditions are shown in Table 1. Indicated transcripts were reverse transcribed and amplified with a commercial system (One Step RT-PCR; Invitrogen, Gaithersburg, MD).

Cellular and Nuclear Extract Preparations

Whole-cell and nuclear extracts of treated and untreated HLE cells were prepared according to a standard protocol. $^{21}\,$

Western Blot Analysis

Whole-cell extracts (40 μ g of total proteins) were analyzed by SDS-PAGE. The anti-catalase antiserum (Abcam, Cambridge, UK), anti- α -1-antitrypsin (Stressgen, Victoria, British Columbia, Canada), and anti-basigin²² (gift of Brian Toole, Tufts University, Boston, MA) were used at a dilution of 1:1000; the secondary antibody, anti-rabbit horseradish peroxidase

(Vector Laboratories, Burlingame, CA), was used at 1:5000 dilution; and chemiluminescence was detected with a kit (Pierce, Rockford, IL). Antibodies against transcription factors were arylhydro-carbon receptor nuclear translocator (ARNT; Novus Biologicals, Littleton, CO); c-fos, c-jun, JunD, and NF- κ B (p50) (Santa Cruz Biotechnology, Santa Cruz, CA); and lens epithelium-derived growth factor (LEDGF; gift of Toshimichi Shinohara, Brigham and Women's Hospital, Boston, MA).

Flow Cytometry Analysis of Apoptotic Cells

Recovery of the cells was monitored by examining the levels of apoptosis at 0, 1, 2, 8, and 24 hours after the H_2O_2 treatment. Annexin V binding and propidium iodide staining were determined by flow cytometry, using reagents from Roche Molecular Biochemicals (Indianapolis, IN). The cells were treated with 50 μ M H_2O_2 for 1 hour, as described earlier, washed with ice-cold PBS, and double stained with FITC-coupled annexin V protein and propidium iodine for 20 minutes. Flow cytometry was performed with a 488-nm laser coupled to a cell sorter (FacsCalibur; BD Biosciences, San Jose, CA). Cells stained with both propidium iodide and annexin V were considered necrotic, and the cells stained only with annexin V were considered apoptotic.²³ The data are based on multiple analyses of two independent experiments.

Results

Cell Survival versus Apoptosis after H₂O₂ Treatment

Multiple concentrations of H_2O_2 were initially tested to determine an H_2O_2 concentration that did not induce significant apoptosis. These experiments indicated that a 1-hour treatment with 50 μ M H_2O_2 resulted in minimal apoptosis or necrosis up to 24 hours after treatment (Fig. 1). The total apoptotic and necrotic cells was 8% compared with 2% in untreated cells. The damaged cells detached from the surface and were removed during the change of medium. Analysis of the remaining cells at 8 and 24 hours subsequent to H_2O_2 treatment demonstrated levels of apoptosis and necrosis that were indistinguishable from that in the control cells.

Induction of Representative Genes Involved in H₂O₂ Detoxification

To determine that 50 μ M H₂O₂ significantly induces gene expression in HLE cells, we evaluated by RT-PCR the transcript levels in treated lens cells of five enzymes known or believed to be essential in H₂O₂ protection, including CuZn and Mn superoxide dismutases (SODs), glutamylcysteine synthetase (γ GCSH), glutathione *S*-transferase theta 2 (GSTT2), and thioltransferase (TTase) (Fig. 2). We detected elevated levels of three transcripts (CuZn and Mn superoxide dismutases, and glutathione *S*-transferase theta 2) at both 1 and 2 hours after the initial treatment. In contrast, transcripts encoding glutamylcysteine synthetase were reduced at 1 hour and elevated at 2 hours after the initial treatment. Because treatment with 50 μ M H₂O₂ yielded significant gene inductions with minimal apoptosis or necrosis, this concentration of H₂O₂ was chosen for large-scale expression profiling of HLE cells.

Induction of Representative Genes Encoding Transcription Factors Typically Involved in H₂O₂ Response

Next, we examined protein levels of several transcription factors— c-Jun, c-fos, Jun D, NF- κ B, ARNT, and LEDGF—all known to be regulated by various forms of stress at multiple levels (e.g., at the level of transcription, posttranslational modification, and subcellular localization).^{6,12,15} The immunoblots are shown in Figure 3. The data showed that as early as 30 minutes after the initial treatment with H₂O₂, levels of the proteins c-Jun, Jun D, c-Fos, and NF- κ B in nuclear extracts were increased, in agreement with known roles of these transcription factors in the immediate response to OS. In contrast, significantly elevated levels

of ARNT and LEDGF were found only at 1 hour after the treatment (data not shown). The increased levels of c-Jun, c-Fos, and Jun-D visible 30 minutes after the initial treatment are attributable to de novo transcription of these genes, mediated by the autoregulatory loops.²⁴ In contrast, the presence of NF- κ B in the nuclear extract after 30 minutes of H₂O₂ treatment resulted from the dissociation of the inactive cytoplasmic complex of NF- κ B and I κ B, followed by nuclear entry of NF- κ B.²⁴ From these data, we conclude that HLE cells treated with 50 μ M H₂O₂ for 1 hour respond to OS by changing the RNA and protein levels of an essential group of transcription factors, demonstrating that HLE cells are widely responsive to H₂O₂ treatment. These data also suggest that major stress pathways were not abrogated in the SR-01-04 HLE cells.

Dynamic Changes in Expression of 1171 Genes that Respond to H₂O₂ Insult

To determine the spectrum and range of genes that respond to 50 μ M H₂O₂ in HLE cells, we conducted a series of dual-color cDNA microarray hybridizations. The experimental design (Fig. 4) included several controls to distinguish effects of serum starvation and stimulation by the 50- μ M H₂O₂ treatment. The reference RNA used was prepared as a pool of three RNAs obtained from treatments 0, 1W (H₂O), and 2WS (H₂O/serum). Pooling multiple RNAs is a standard practice in global gene expression studies, to enhance detection of biologically significant genes.^{25,26} The reference RNA in this study was always labeled with Cy3 substrate, and the tested samples (0, 1P [H₂O₂], 2PS, 8PS, 2P, 1W, 2WS, and 8WS) were labeled with Cy5 substrate. Reverse labeling was not conducted, because the data analysis was performed using the SAM algorithm (as described in the Methods section) to evaluate expression of each gene for its deviation from all other time points and experimental conditions.

Using the SAM analysis, 20 we evaluated results of three individual experiments: H₂O₂/serum, H₂O₂, and H₂O/serum (Fig. 4). The H₂O₂/serum pathway (0-1P-2PS-8PS) was a primary experiment to identify differentially expressed genes that respond to OS treatment. In contrast, the pathway 0-1W-2WS-8WS was a control experiment (H₂O/serum), to eliminate the possibility that changes in gene expression result from serum treatment alone. To further distinguish true H₂O₂ gene induction from serum induction, a third pathway, H₂O₂ (0-1P-2P) representing OS caused by prolonged serum starvation, was also studied. Because the overwhelming majority of transcript levels at 8 hours after treatment were similar to those in the control cells and our focus was on primary-response genes, the 8-hour time point was excluded from the SAM analyses. In the H₂O₂/serum and H₂O₂ pathways, we found, respectively, 823 and 219 differentially expressed genes unique to each pathway. Differential expression of 129 transcripts was common to both courses of treatment. Altogether, 1171 statistically reliable, differentially expressed transcripts were detected, with a false discovery rate of less than 1%.

The time-course display of the relative intensities of these 1171 genes is shown in Figure 5. Individual genes are displayed as dots. The pattern of dots from each experiment indicates the amplitude of changes of the individual transcript levels. The H_2O_2 /serum (Fig 5, top) diagram contains 823 unique and 129 common genes: 952 individual dots; H_2O_2 (Fig. 5, middle) contains 219 unique and 129 common genes: 348 individual dots and H_2O /serum (Fig. 5, bottom) contains all 1171 genes and therefore the same number of dots. The zero time point represents the ratios of untreated cells divided by the pooled reference. After 1 hour of treatment with 50 μ M H_2O_2 , followed by cultivation in the presence of serum, transcript levels diverged into two distributions, representing up- and downregulated transcripts. Two hours after the initial treatment, a visible majority of transcripts returned to their original levels. Nevertheless, a significant proportion of transcripts (n = 237) were still upregulated by at least a factor of 1.5 ($n_1 = 206$ genes), or downregulated by at least a factor of 0.67 ($n_2 = 31$ genes). Eight hours after the initial treatment, the patterns indicate that the overwhelming majority of transcript

levels returned to the levels in the control cells. By contrast, in the H_2O_2 pathway, the split between up- and downregulated genes was maintained at 2 hours after the initial treatment. In the control experiment (0-1W-2WS-8WS), transcript level changes were less dramatic and reflect the 1 hour of serum starvation followed by stimulation with serum. The normalized 8hour time point of the H_2O_2 /serum expression pattern and the minimal levels of apoptotic cells suggest that the H_2O_2 -treated cells in the H_2O_2 /serum pathway were approaching the normal state.

Functional Classification of OS-Responsive Genes

Gene clustering based on the expression profiles revealed seven types of responses (Fig. 6). To annotate the list of 1171 genes according to their function, we performed comprehensive database searches. We used a 12-group classification system to assign putative function to 43% (n = 509) of the genes.²⁷ The groups are (Fig. 7) (1) cell growth, division, and DNA synthesis; (2) cell rescue, defense, and death; (3) cellular organization; (4) energy and mitochondria; (5) intracellular transport; (6) ionic homeostasis; (7) metabolism; (8) protein synthesis and degradation; (9) signal transduction; (10) splicing; (11) transcription; and (12) transport facilitation. We added two groups, one containing annotated genes without any deduced function and the other containing ESTs. Some genes can be classified in at least two categories. The prime examples are regulatory kinases and phosphatases that may belong to both groups 1 (81 genes) and 9 (72 genes), as illustrated in Figure 7. Five genes from these categories were accounted for in two categories. The total number of genes in these categories that were present on the microarray was calculated from data provided by Mariadason et al.²⁸ The presence of a number of significantly regulated genes in each category indicates that OS affects all 12 functional groups.

Secondary Confirmation of Microarray Results

To provide confirmation of transcript level differences resulting from the H₂O₂ treatment, a series of semiquantitative RT-PCR analyses was conducted with specific primers used to detect five representative genes: CD47, amyloid β A4 precursor protein (APP), β -tubulin (TUBB), Jun NH₂-terminal kinase (JNK) interacting protein 2 (MAPK8IP2), UDP-galactose: β -*N*-acetylglucosamine β -1,4-galactosyltransferase 1 (B4GALT1), and Fanconi anemia complementation group A (FANCA). CD47 was selected from the group of unchanged genes and analyzed at 0, 1, 2, 3, 5, and 8 hours. The results (Fig. 8) confirm the trends in transcript levels of specific genes initially detected by the microarray study. These trends are also likely to be reflected at the protein level, because increased catalase (group 2, cell rescue, defense, and death), α -1-antitrypsin (group 8, protein synthesis and degradation), and basigin (group 3, cellular organization) were also detected at the protein level (Fig. 8D). These proteins were selected because of the availability of specific antibodies and because they represent different functional groups.

We did not examine transcript levels of additional genes (e.g., transcripts repressed by the OS treatment) with semi-quantitative RT-PCR, because we were mainly interested in genes that are upregulated with OS treatment and are therefore likely participants in the defense against OS. It was interesting that from the group of five genes encoding antioxidant enzymes, only one spot for glutamylcysteine synthase (γ GCSH) was present on the array. However, this transcript was not analyzed by SAM, because five data entries from 27 chips used did not pass through the initial data-filtering step. Future studies will be undertaken to compare directly the present microarray data with the absolute magnitude of change of most biologically interesting transcript levels at an expanded number of time points, to identify peak values and respective time points of each transcript by real-time RT-PCR.

Discussion

Analysis of Smaller Categories of OS-Responding Genes

OS-related damage to the genomic DNA, if not successfully repaired, will ultimately trigger apoptosis.²⁹ H₂O₂ and reactive oxygen species are decomposed by various antioxidant enzymes. Cells under OS stress respond at the level of de novo protein synthesis, identifying damaged proteins followed by degradation and/or repair, involving their refolding. Thus, we used database searches to classify genes into individual smaller categories according to function, including DNA repair, molecular chaperones, antioxidant enzymes, ribosomal and accessory proteins, protein trafficking, protein sorting, secretion and quality control, proteasome degradation, and proteinases and their inhibitors. The expression profiles used to determine similarly regulated genes were developed with self-organizing maps from the MultiExperiment Viewer software. Transcript levels are displayed in a seven-color system (Fig. 9). Our method of data presentation and analysis is based on grouping proteins according to their functions, analyzing their responses to OS, and looking for functionally similar genes present on the microarray that do not respond to the OS. We do not believe there is a direct correlation between function and the magnitude of change in gene expression, because many biological processes are regulated by a twofold change.²⁰ In addition, a series of twofold changes in the activities of enzymes operating in the same pathway are likely to be amplified in term of biological responsiveness.

OS and DNA Repair

The DNA repair subcategory includes 11 regulated genes, whereas 5 additional genes in this category appear not to be regulated by OS in the present system. Transcripts encoding FANCA, ATDC, RAD23B, BRCA2, and FANCG were specifically upregulated by OS between 1 and 2 hours after H₂O₂ treatment (Fig. 9). In contrast, the remaining six transcripts encoding TH1, MLH1, PRKDC, RAD21, APEX, and BRCA1 were repressed by OS. Induction of FANCA, FANCG, and BRCA1 by OS has not been described. It should be noted that the genes FANCA, FANCG, BRCA1, and BRCA2 may be components of the same DNA repair pathway, as judged from their subnuclear colocalization in previous studies³⁰; however, the precise function of these genes is not currently known.³¹

Molecular Chaperones and Chaperone-like Proteins

The molecular chaperone group contains many genes known to be induced by OS and other forms of stress. Our data show significant upregulation of eight transcripts encoding classic heat shock proteins, chaperones involved in β -tubulin folding, and β -tubulin (TUBB; Fig. 9). Elevated levels of transcripts encoding ORP150 and β -tubulin were found both at 1 and 2 hours after OS treatment. Other members of this group were either initially activated (HSC70, TBCC) or decreased at 1 hour and upregulated at 2 hours (DYT1, VBP1, and CCT8). Five transcripts encoding proteins with chaperone and chaperone-like functions (PFDN4, TR1/gp96, CCT4, CCT7, and HSP10) did not respond to the OS treatment. Prefoldin 4 (PFDN4) was upregulated (by a factor of 1.3) only at 8 hours. OS caused prolonged induction of TUBB, RP2, CCT2, HSC71, HSP40, and ORP150 in the absence of serum (Fig. 9). ORP150 is a widely studied protein that provides protection to neurons from ischemic stress³² and is not known as an OSinduced gene. Serum stimulation of the cells specifically induces DYT1, a member of the AAA + family of molecular chaperones.³³ The heat shock proteins, HSP70 and HSP90 prevent the formation of apoptosome from Apaf-1, pro-caspase-9 and cytochrome $c.^{34,35}$ Our present data raise the possibility that functionally similar proteins, HSC70 and HSP40, both induced by OS already at 1 hour are also engaged in this process. ORP150 is known as a potent inhibitor of caspase-3.³² OS induction of transcripts that encode molecular chaperones, such as ORP150, retinitis pigmentosa 2,³⁶ and β -tubulin, raises the possibility that these proteins have a broad stress-protective function in the lens epithelium.

Enzymes Involved in the Metabolism of Glutathione and Antioxidant Enzymes

Eleven transcripts encoding various glutathione S-transferases were monitored, but only two of them (MGST2 and GSTM3) showed significant changes in their expression profiles according to SAM, whereas the remaining genes (GSTM5, GSTP1, GSTTLp28, GSTT1, GSTA3, GSTZ1, GSTA4, GSTM4, and MGST1) were not identified as statistically significant (Fig. 9). Transcripts from genes encoding glutathione peroxidase 1 and 4 and glutathione synthetase were also not changed under the present experimental conditions. Five antioxidant and detoxifying enzymes (paraoxonase 2, thioredoxin reductase, ATOX1, and peroxiredoxin 2 and 3) were selected by SAM, whereas two genes (catalase and ALDH6) did not pass the criteria for significance and/or quality of the raw data. Inspection of the raw data combined with RT-PCR and Western analysis results (Fig. 8) caused us to conclude that catalase was induced by 50 µM H₂O₂ in our experiments. Most of the transcripts were unexpectedly repressed at 1 hour after treatment, with the exception of thioredoxin reductase, which was upregulated in both serum-free and serum-stimulated conditions. Thus, the data suggest that constitutively expressed glutathione S-transferases are sufficient to defend cells against the stress induced in the current model and that peroxiredoxins 2, -3, and -5, together with catalase and GSTT2, serve as antioxidative enzymes. With the exception of catalase, we did not find any significant overlap between genes induced by a defined 1-hour treatment with 50 µM H₂O₂ and genes with expression and/or copy number that was increased in mouse lens cells resistant to the high levels (125 μ M H₂O₂) of chronic OS identified by Spector et al.³⁷ However, a direct comparison of these two data sets is limited because different microarrays were used.

Ribosomal Proteins and Elongation Factors

Three transcripts encoding ribosomal subunits—L18, L29, and S15—were specifically repressed by OS (Fig. 9). In contrast, the S19 subunit was activated by OS. Three transcripts encoding elongation factors EEF2, EIF3S9, and EIF5G1 showed robust induction caused by OS. Four genes encoding EIF1 ϵ 1, EIF1AY, EIF2 γ 3, and mitochondrial MTIF2 were selectively repressed by OS.

Protein Trafficking, Secretion, and Quality Control

The protein trafficking subcategory is composed of two subclasses, including classic proteins involved in protein trafficking and specialized proteins (nuclear import subclass) facilitating import of other proteins into the nucleus (Fig. 9). Both groups contain early-response (e.g., metaxin 1, KPNB1, and RABL2B) and delayed-response (e.g., ARF3, ARF5, and ARF4L) genes and contain numerous genes with highly upregulated transcripts (ARF1, ARF3, and KPNA2). Five genes in the subcategory protein sorting, secretion, and quality control showed significant changes in their expression profiles. Transcripts encoding AP3D1 and VPS26 are involved in protein sorting. SEC6 and NPTX1 are involved in protein secretion, and calreticulin controls the quality of newly synthesized proteins. Calreticulin also exhibits a significant antiapoptotic activity.³⁸ Several members of this subcategory (calnexin, SEC61B, SEC63L) that were present on the chip were not affected by 50 μ M H₂O₂-induced OS. Transcripts encoding SEC61G were induced by a factor of 1.7 only at 8 hours.

Proteasome and Ubiquitination Pathways and Other Routes of Protein Degradation

The majority of transcripts encoding various proteins involved in proteasome-mediated degradation of proteins were reduced as a specific result of imposed OS (Fig. 9). The notable exception was FBXO3, a ligase component of the ubiquitination pathway. Transcripts encoding 14 proteinases and 6 inhibitors of proteinases were found among the 1171 genes. The most notable transcript encoding a proteinase inhibitor was α -1-antitrypsin, induced by OS (Fig. 8D). Induction of amyloid β A4 precursor protein (APP) by OS in the lens has been shown.

 39 Neither caspase-9 nor -3, both enzymes in the top of the mitochondria-directed apoptotic cascade, was among the significantly changed genes. 35 The conclusion drawn from expression profiles of these genes is that they are involved in processes favoring cell survival (e.g., α -1-antitrypsin has strong antiapoptotic activity), 40 elimination of damaged proteins (e.g., FBX03 belongs to a protein ligase complex promoting ubiquitination and degradation), and mobilization of translational machinery (e.g., increase of transcript levels for elongation factors EEF2 and EIF3S9), in parallel with apparent activation of processes used during the apoptosis.

OS and Other Categories of Genes

The remaining functional categories were also analyzed in a format identical with Figure 9 and the data are displayed on our Web site (www.aecom.yu.edu/thecvekllab).

OS and Cataract

Our previous studies have identified a group of differentially expressed genes in human cataractous epithelia^{8,10,41} and in an Emory mouse model of age-related cataract.⁴² A 55kDa regulatory subunit (R2/B/PR55) of the protein phosphatase 2A (PP2A) is downregulated in human cataractous epithelia.⁸ PP2A comprises two invariant subunits, A and C, combined with three different regulatory B subunits encoded by separate genes. PP2A is a major phosphatase that regulates a variety of key steps in metabolism, replication, transcription, and cell-cycle control. Recent studies have shown involvement of PP2A in various damage-sensing pathways, including the ionizing radiation.⁴³ Our data show that H₂O₂ induces expression of the R3/B"/PR72 subunit while reducing expression of the scaffolding subunit A (Fig. 9), raising the possibility that the PP2A-pathway is involved both in the age-related cataract and OS response. More recent data suggest that expression of genes encoding ribosomal subunits (L21, L15, L13a, and L7a) is reduced in cataractous epithelia.⁴¹ Our present data indicate downregulation of subunits L18, L29, and S15. Genes encoding L21, L15, and L7a exhibit approximately 25% reduced expression at 1 and 2 hours after the treatment. Because of the strict parameters of SAM, these genes did not qualify for the list of 1171 genes significantly affected by H₂O₂ treatment. The present data validated by RT-PCR (data not shown) also show H₂O₂ induction of transcript levels encoding a receptor tyrosine kinase ARK/AXL that correlates with the specific upregulation of this gene in the Emory cataract mouse model.⁴²

OS induces levels of two enzymes, B4GALT1 and MVK (Fig. 9). B4GALT1 encodes an enzyme that uses UDP-galactose as a substrate.^{22,44} UDP-galactose is formed by GALT1, a central enzyme in galactosidase metabolism. Mutations in GALT1 cause congenital cataracts, ⁴⁵ and disturbed galactose metabolism in elderly and diabetic humans is associated with cataract.⁴⁶ Two more proteins participating in galactose metabolism through glycosylations, LGALS3 and FUT2, are also affected by OS in lens cells. Defects in cholesterol biosynthesis can also cause cataracts. Mutations in mevalonate kinase are associated with congenital cataracts, ⁴⁷ and mevalonate kinase is also induced by OS.

In conclusion, our study provides a representative global view of the acute response to OS in HLE cells. In contrast to several similar studies of OS and different cell culture models analyzing the apoptotic pathways, 48,49 our study focused on successful defense mechanisms. We found multiple novel responses to OS in HLE cells and identified numerous genes for future functional studies. Our data support the idea that OS changes the levels of those specific transcripts and gene expression pathways likely to be involved in lens protection and cataract. $^{4-6,37,49}$ These transcriptional responses point to candidate genes, the study of which will lead to further understanding of and development of therapies for prevention of numerous OS-and age-related diseases. 51

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Flow cytometry analysis of the proportion of apoptotic and necrotic cells followed a 1-hour 50- μ M treatment with H₂O₂.



Figure 2.

Expression of representative enzymes detoxifying OS. Semiquantitative RT-PCR analysis of CuZn superoxide dismutase (Cu/Zn-SOD), glutamylcysteine synthetase (γ GCSH), glutathione *S*-transferase theta 2 (GSTT2), Mn superoxide dismutase (Mn-SOD), and thioltransferase (TTase) in human lens epithelial cells treated with 50 μ M H₂O₂ for 1 and 2 hours, and control (0) human lens epithelial cells. The primers, sizes of products, and annealing temperatures are in Table 1.



Figure 3.

Western blot analysis of protein levels of transcription factors c-Jun, c-fos, Jun D, and NF- κ B known to be regulated by redox state. Time (in hours) and relative molecular mass (in kilodaltons) are indicated.



Figure 4.

Experimental design of the treatment of HLE cells with 50 μ M H₂O₂. Hybridizations were conducted with RNA isolated from indicated experiments (*circles*). Reference RNA was prepared as a 1:1:1 (wt/wt/wt) pool from three experiments, 0, 1W, and 2WS.



Figure 5.

Significant changes in transcript levels of panels of genes responding to the treatment of HLE cells responding to the 50- μ M H₂O₂ treatment for 1 hour (Fig. 4). Diagram represents of distribution of normalized signal intensities of tested versus common reference (pooled) samples. The genes included were only the 1171 that met the SAM criteria for significance of up- or downregulation. *Top*: human lens epithelial cells treated with 50 μ M H₂O₂ (H₂O₂/serum pathway). *Middle*: cells treated with 50 μ M H₂O₂ followed by 1 hour of serum starvation, (H₂O₂ pathway). *Bottom*: control cells (H₂O/serum pathway). Triplicates are shown.



Figure 6.

Seven representative expression profiles. The expression profiles are based on data from 0-, 1-, and 2-hour time points that yield a maximum of nine patterns of expression. Seven patterns, labeled A, B, C1, C2, D1, D2, and E, were detected using Genecluster. Clusters B, C1, C2, D1, D2, and E represent early-response genes and cluster A represents delayed-response genes.



Figure 7.

Summary diagram showing functional categories of 1171 genes selected by SAM. The genes regulated by OS were classified into 12 functional groups according to Gerstein and Jansen. ²⁴ The diagram of genes spotted on the microarray, shown for comparison was generated using independently collected annotations by Mariadason et al.²⁸ Genes in group 6, ionic homeostasis, were not classified separately.

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Figure 8.

Data validation of a selected group of genes at the transcript and protein levels. (**A**) Semiquantitative RT-PCR analysis of CD47 transcript levels used as a control for the unchanged gene. (**B**) Semiquantitative RT-PCR analysis of transcript levels of amyloid β A4 precursor protein (APP, group 8), β -tubulin (TUBB, group 3), Jun NH₂-terminal kinase (JNK) interacting protein 2 (MAPK8IP2, groups 2 and 9), UDP-galactose: β -*N*-acetylglucosamine β -1,4-galactosyltransferase 1 (B4GALT1, group 3), and Fanconi anemia complementation group A (FANCA, group 1). The primers, sizes of products, and annealing temperatures are given in Table 1. Functional groups are listed in Figure 7. (**C**) Semiquantitative RT-PCR analysis of catalase (group 2) transcript levels. On left, DNA ladders are shown in (**A**), (**B**), and (**C**). (**D**) Western blot analysis of catalase (group 2), α -1-antitrypsin (group 8), and basigin (group 3).

1.1.1.1.1.1	Press and the second seco		Peros	ALC + S	erum		1.eto)	the one		-	Contr	01	
	Gene name (common name)	Асс во	0	1	2	8	0	1	2	0		2	8 hrs
	Panconi anemia, complementation group A (PANCA)	AA644 129	0.0	1000	10	0.9	0,0	10000	0,7	0.6	1.0	0.9	1.0
	ataxia-teangiectasia group D-associated protem (ATDC)	AA035486	11	14	1.8	10	11	14	12	u	13	0.0	1.0
1	RAD23 (8. cerevisiae) homolog B (RAD23B)	N35067	LI	17	1.2	0.6	11	1.7	14	11	1.4	0.7	0.9
-	breast cancer 2, early onset (BRCA2)	148 12 2	11	1.9	1.2	0.8	11	1.9	17	11	1.2	1.0	0.9
8	Panconi anemia, complementation group G (FANCG)	AA430675	11	0.5	1.6	1.4	- 11	0.5	0.8	11	0.9	1.0	1.0
2	THI drosophila homolog (HSPC 130)	AA004210	1.0	0.5	0.8	0.9	1.0	0,5	0.3	1.0	11	0.6	1.1
Z	mutL (E. coli) homolog 1 (colon cancer, nonpolyposis type 2) (MLHI)	R 10662	1.0	0.5	0.8	1.0	10	0.5	0.2	1.0	0.9	0.5	1.1
9	protein kinase, DNA-activated, catalytic polypeptide (PRKDC)	R 2 76 15	0.9	0.4	0.9	0.7	0.9	0.4	0.3	0.9	0.9	0.8	1.0
	RAD21(S. pombe) homolog (RAD21)	AA683 102	0.9	0.4	0.7	0.9	0.9	0.4	0.2	0.9	1.1	0.3	1.2
	APEX nuclease (multifunctional DNA repair enzyme) (APEX)	AA478273	1.1	0.5	0.9	1.2	1.1	0.5	0.4	1.1	0.8	0.8	1.1
	breast cancer I, early onset (BRCAI)	H90415	1.1	1.8	1.0	1.1	1.1	1.8	2.0	11	1.4	0.8	1.1
_							-	-	_		-	-	
	tubum, beta potypeptale (108B)	AA427899	12			12	12			1.2	1.1.1	1. Sec. 1	8.0
	oxygen regulated protem (OKP B0)	AA099134	1.0	1.2.2		0.9	10	1000		1.0	0.9	1.0	0.8
	reimitis pigmentosa 2 (RP2)	W00899	11		11	0.7	11			ш	- 13	0.7	1.1
- E	chaperonin containing TCP1, subunit 2 (CCT2)	N38959	LI	17	10	1.0	1.1	1.7	1.4	11	1.2	0.7	1.0
2	tubulin-specific chaperone c (TBCC)	AA954188	0.8	1.8	10	0.9	0.8	1.8	1.1	0.8	1.4	1.2	1.0
2	heat shock 40kD protein 1(HSPF VHSP40)	AA435948	1.0	17	1.9	1.0	1.0	1.7	1.3	1.0	1.0	0.6	1.1
E.	heat shock 70kd protein 10 (HSC 70)	AA629567	1.1	1.7	0.6	1.6	11	1.7	1.1	1.1	0.5	1.8	1.2
	N-ethylmaleimide-sensitive factor (NSF)	H97488	0.9	0.4	0.6	0.8	0.9	0.4	0.2	0.9	0.9	0.6	1.0
	FK506-binding protein 5 (PKBP5)	W86653	1.0	0.5	0.9	0.7	1.0	0.5	0.3	1.0	1.0	0.5	0.9
	dystonia 1, torsion (DYTI)	AA394148	11	0.6	13	0.9	11	0.6	0.6	1.1	0.9	1.2	1.2
	von Hippel-Lindau binding protein I(VBPI)	AA426341	0.9	0.4	0.8	1.1	0.9	0.4	0.3	0.9	0.8	0.6	1.1
	chaperonin containing TCP1, subunit 8 (CCT8)	AA630016	1.0	0.5	0.8	13	1.0	0.5	0.4	1.0	1.0	0.5	1.1
	dynein, cytoplasmic, light polypeptide (PIN)	AA644679	1.2	0.5	0.9	1.4	1.2	0.5	0.4	12	0.8	0.9	1.4
-			-							-			
	cysteine desulfurase (NIFS)	AA476245	1.2	0.6	0.9	0.9	1.2	0.6	0.5	1.2	0.8	0.8	1.2
	ATX1 (antioxidant protein 1, yeast) homolog 1(ATOX1)	AA4 18755	11	0.5	1.0	0.9	11	0.5	0.5	1.1	10	0.8	1.0
4	microsomal glutathione S-transferase 2 (MGST2)	W73474	11	0.6	13	0.8	11	0.6	0.6	1.1	1.0	0.8	1.1
5	glutathione S-transferase M3 (GSTM3)	R63106	1.0	0.4	1.0	0.9	10	0.4	0.2	1.0	11	0.6	LI
3	paraoxonase 2 (PON2)	AA446301	10	0.4	L	10	10	0.4	0.3	1.0	12	0.6	11
3	peroxiredoxin 3 (PRDX3)	H19203	0.9	0.4	0.8	10	0.9	0.4	0.1	0.9	12	0.5	12
	thioredoxin reductase beta (TR)	AA414130	10	100	1000	0.9	10	1000	12	10	0.9	0.8	0.8
	peroxiredoxin 2 (PRDX2)	HERRAS	12	0.9	1.0	14	12	0.9	10	12	1015	0.6	1.5
		INCOLD		0.3	-	-		0.5	1.0		-	0.0	-
-27-	translation initiation factor 3, subunit 9 (EIF3S9)	AA676471	11	16	Contract of the local division of the local	10	11	16	14	11	15	0.5	10
	ribosomal protein S19 (RPS19)	H4 116 5	10	200	13	12	10	12.0	1.4	10	14	0.8	10
-	translation initiation factor 4 gamma 1(FF4Gb	R 3 72 76	10	11		10	10	11	0.9	10	11	0.8	0.9
-	ribosomal protein Lis (RPLIS)	A 4 7769 74	11	0.0		0.5	11	0.5	0.0		0.0	0.0	11
e	above mail protein (A) (AD(20))	A 10 18 (13	0.0	0.7	1.1	0.0	0.0	0.7	0.9		0.9	0.9	
2	above mal protein (29) (KP129)	ADISCIS	0.9	0.7	10	0.6	0.9	0.7	0.9	0.9	0.7	0.7	0.9
-	ribosoma protem (S15)	N 59 53 4	10	0.7	and the second	0.0	10	0.7	12	1.0	0.7	0.9	10
1	translation elongation factor l'epsilon I (EB' le I)	N394 B	LO	0.7	0.0	0.9	10	0.7	0.4	1.0	ш	0.8	1.1
	translation initiation factor 1A, Y chromosome (EIF IAY)	AA047039	11	0.5	0.7	0.8	11	0.5	0.4	11	0.9	0.8	1.1
4	mitochondrial translational initiation factor 2 (MTIF2)	H18070	1.1	0.6	0.8	0.6	1.1	0.6	0.4	1.1	0.8	0.7	1,1
×	translation initiation factor 2, subunit 3 (EIF2g3)	AA454581	0.9	0.5	0.7	0.9	0.9	0.5	0.3	0.9	0.9	11	1.2
-	P. hav and anothin 1 (FROVI)	N170 4 17							1.00				10
	P-box only protein 3 (PBOX3)	N70417	11		-	0.9	_ 11		1.1	11	1.2	0.8	1.0
	ubiquitin-conjugating enzyme E2E 2 (UBE2E2)	AA453584	0.8	1.8	0.8	0.9	0.8	1.8	0,8	0.8	1.1	10	0.9
	ub iquit m-conjugating enzyme E2G 1(UBE2G1)	AA113881	11	0.6	11	10	- 11	0.6	0.7	u	1.2	0.9	1.1
S	proteasome 26S subunit, ATPase, 3 (PSMC3)	AA282230	11	0.7	14	1.0	- 11	0.7	0.7	- 11	13	0.6	1.1
3	proteasome 26S subunit, non-ATPase, 3 (PSMD3)	AA485052	1.0	1.0	1.6	0.8	1.0	10	0.8	1.0	1.0	0.7	0.9
2	proteasome subunit HsC7-I, complete cds (HsC7-I)	N73252	LI	0.5	0.9	0.8	11	0,5	0.3	11	1.2	0.7	1.0
-	ubiquitin carrier protein (E2-EPF)	AA464729	0.9	0.5	1.1	1.2	0.9	0.5	0.3	0.9	0.8	0.9	1.1
	ubiquitin specific protease 16 (USP16)	AA489619	1.0	0.4	0.7	0.9	1.0	0.4	0.4	1.0	0.9	0.8	1.1
	proteasome subunit, alpha type, 1 (PSMAI)	R27585	1.0	0.3	0.9	1.3	1.0	0.3	0.3	1.0	1.0	0.7	1.1
_							-	_			-		
	metalloprotease I (MPI)	AA447691	1.0	1.00	0.5	0.8	1.0	1000	0.9	1.0	0.7	18	11
	serme protease inhibitor, Kunitz type 1 (SPINTI)	AA975209	-0.5	1.8	1.0	0.7	- 0,5	1.8	0.7	0.5	0.8	1.1	0.9
	Homo sapiens mRNA for neurotrypsin (neurotrypsin)	AA683308	1.1	2.1	1.5	0.8	11	2.1	2.1	11	1.5	0.7	1.1
2	amyloid beta (A4) precursor protein (APP)	W42849	0.9	2.1	1.9	11	0.9	2.1	2.0	0.9	2.4	0.4	0.7
5	a 1-antitrypsin	H77595	11	11	13	0.9	1.1	2.1	2.0	1.1	1.4	0.7	1.1
4	cathepsin B (CTSB)	W47179	1.0	1.9	1.1	0.7	1.0	1.9	2.0	1.0	1.3	0.7	1.1
4	caspase 8, apoptosis-related cysteine protease (CASP8)	AA448468	11	1.8	11	13	1.1	1.8	1.8	1.1	1.2	0.9	1.2
-	quiescent cell proline dipeptidase (QPP)	AA4 18 3 9 5	11	13	3.0	1.0	11	13	1.0	1.1	1.0	0.7	0.9
P	endothelin converting enzyme-like 1 (ECEL)	R61395	0.9	12	1.2.2	0.9	0.9	12	12	0.9	1.0	0.6	0.8
	calnain 4 small subunit (CAPN4)	AA676484	13	0.7	3.0	0.9	11	0.7	0.5	11	16	0.4	12
1 Ke	protective protein for beta-galactosidase (PPGB)	AA916327	10	0.7	14	0.8	10	0.7	0.6	10	14	0.9	0.8
-	protessa inhidar 0 (PB)	4410513	10	07			1.2	0.7	0.4	12	0.0	0.6	
ote	procellagen bying 2 over bitarate 5 dis yearnase (PLOD)	A 4 76 3 40	12	0.7	12	10	10	0.7	0.4	12	11	10	1.2
E	leucine amino nantidasa (LOC \$0.56)	AA476240	1.2	0.1	12	10	- 12	0.7	0.4	12	11	LO	12
-	eucuse anuno peptidase (LLR, SID36)	R69307	11	0.6	8.0	8.0	- 11	0.6	0,4	11	0.8	0.9	11
	protease mnin nor 2 (anti-elastase), monocyte/neutrophil (ELANH2)	R 54664	1.0	0.4	0.9	0.9	LO	0.4	0.4	1.0	10	0.8	0.9
	Prothymosin alpha (Prothymosin)	H48420	11	0.4	1.0	0.7	11	0.4	0,4	11	0.9	0.6	13
	cathepsin L (C TSL)	W73874	1.1	0.5	13	1.5	11	0.5	0.5	11	LI.	0.8	1.2
	N-acylaminoacyl-peptide hydrolase (APEH)	AA456408	1.2	0.4	1.2	0.9	1.2	0.4	0.2	1.2	1.0	0.8	1.2
1000	cathepsin C (CTSC)	AA644088	1.0	0.4	13	1.0	1.0	0.4	0.3	1.0	1.2	0,6	1.1
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	ADP-ribosylation factor 1 (ARFI)	W45572	0.9	1.8.8	1201	0.8	0.9	1000	2.0	0.9	U	0.5	0.7
	RAB36, member RAS oncogene family (RAB36)	H69004	1.0	19	1.2	0.9	1.0	1.9	1.9	10	1.3	0.8	1.2
	metaxin I	N69540	1.2	1.8	0.9	1.1	1.2	1.8	1.6	1.2	0.9	1.6	13
	RAB, member of RAS oncogene family-like 2B (RABL2B)	AA428182	1.2	2.0	13	13	1.2	2.0	17	1.2	13	0.7	1.0
	adaptor-related protein complex 3, sigma 2 subunit(AP3S2)	R33031	1.2	1.1	13	11	1.2	-2.3 -	2.9	1.2	13	0.9	1.1
25	RAN binding protein 2 (RANBP2)	N77205	1.2	2.0	- 1.1	1.1	1.2	2.0	14	1.2	1.1	0.9	1.0
cing		H15085	0.9	0.6	18	0.6	0.9	0.6	0.7	0.9	0.8	0.8	1.0
fficing	ADP-ribosylation factor 4-like (ARF4L)			10	2.0	0.6	11	1.0	0.9	11	0.8	0.5	1.0
rafficing	ADP-ribosylation factor 4-like (ARF4L) ADP-ribosylation factor 3 (ARF3)	AA670422	1.1	1.0		the second se	-	0.0	10	-	10	11	12
Trafficing	ADP-ribosylation factor 4 -like (ARF41) ADP-ribosylation factor 3 (ARF3) ADP-ribosylation factor 5 (ARF5)	AA670422 AA629584	11	0.9	19	0.8	1.1	0.9	1.0	1.1	10		
cin Trafficing	ADP-robosylation factor 4-like (ARF41) ADP-robosylation factor 3 (ARF3) ADP-robosylation factor 5 (ARF3) ADP-robosylation factor 5 (ARF3) EB-55kDn-associated proteins 5 (EB-AP5)	AA670422 AA629584 AA464 J98	13	0.9	1.9	0.8	11	11	10	11	0.9	0.8	10
otein Trafficing	ADP-rbosylation factor 4 - kite (ARF4L) ADP-rbosylation factor 3 (ARF3) ADP-rbosylation factor 5 (ARF3) EIB-558Da-associated protein 5 (EIB-AP5) karvenberin able 2 (ZIPA2)	AA670422 AA629584 AA464198	11	0.9	19 17	0.8	11	11	10	11	0.9	0.8	1.0
Protein Trafficing	ADP-rhosylation factor 4 - Alae (ARF41) ADP-rhosylation factor 3 (ARF3) ADP-rhosylation factor 3 (ARF3) EB-SSDB-assessicated proteins 6 (EB-AP5) haryophern alpha 2 (EFNA2) haryophern alpha 2 (EFNA2)	AA670422 AA629584 AA464198 AA676460	13 13 12	0.9	19	0.8 0.8 11	11	11	10	13 11 12	0.9 L1	0.8	10 0.9
Protein Trafficing	ADP-reboylation factor 4 - Alas (ARF41) ADP-reboylation factor 3 (ARF3) ADP-reboylation factor 3 (ARF3) EB-53DD-attesticated proteins 5 (EB-AP5) karyopherin alpha 2 (GPAA2) karyopherin (apha 2 (GPAA2) karyopherin (apha 2 (GPAA3)	AA670422 AA629584 AA464 198 AA676460 AA919053	13 13 12 11	0.9 11 0.7 0.7	19 17 30 0.8	0.8 0.8 11 12		11 0.7 0.7	10 10 0.5 0.4		0.9 L1 0.9	0.8	10 0.9 10
Protein Trafficing	ADP-rhospitation factor 4 - Alas (ARF41) ADP-rhospitation factor 3 (ARF3) ADP-rhospitation factor 3 (ARF3) EB-SSDB-assessicated proteins 6 (EB-APS) karyopherin aphae 2 (EWAA2) karyopherin aphae 2 (EWAA2) karyopherin (aphae 2 (EWAA2) ADP-rhospitation factor-16 (EWHB3) ADP-rhospitation factor-16 (EWHB3)	AA670422 AA629584 AA464198 AA676460 AA919053 H20652	13 13 11 12 11 10	0.9 11 0.7 0.7 0.5	19 17 0.8 12	0.8 0.8 11 12 0.8	11 12 11 10	11 0.7 0.7 0.5	10 0.5 0.4 0.5	11 12 11 10	0.9 11 0.9 12	0.8 0.4 0.6 0.7	10 0.9 10 12
Protein Trafficing	ADP-rhosylation factor 4 - Alas (ARF41) ADP-rhosylation factor 3 (ARF3) ADP-rhosylation factor 3 (ARF3) EII-S3DD-arcosticated proteins 5 (EIB-AP5) Euryopherin alpha 2 (OPNA2) Euryopherin (appention) beta 1 (OPNB3) ADP-rhosylation factor-blac 6 uniteracting protein (ARL6 P) Euryopherin (appention) beta 1 (OPNB1) Daryopherin (appention) beta 1 (OPNB1) Daryopherin (appention) beta 1 (OPNB1) Euryopherin (appention) beta 1 (OPNB1) Euryo	AA670422 AA629584 AA464198 AA676460 AA919053 H20652 AA251527	11 13 11 12 11 10 0.9	0.9 11 0.7 0.7 0.5 0.5	19 17 0.8 12 0.9	0.8 0.8 11 12 0.8 10	11 12 11 10 0.9	11 0.7 0.7 0.5 0.5	10 10 0.5 0.4 0.5 0.3	11 12 11 10 0.9	0.9 L1 0.9 L2 L0	0.8 0.4 0.6 0.7 0.6	10 0.9 10 12 11
Protein Trafficing	ADP-rhosylation factor 4-Alas (ARF41) ADP-rhosylation factor 3 (ARF3) ADP-rhosylation factor 3 (ARF3) EB-SSDB-assessicated proteins 6 (EB-APS) karyopherin alpha 2 (KPNA2) karyopherin (approting) beta 3 (KPNB3) ADP-rhosylation factor-like 6 interacting protein (ARL63P) Karyopherin (apporting) beta 1 (KPNB3) ADP-rhosylation factor-like 6 interacting protein (ARL63P) Karyopherin (apporting) beta 1 (KPNB3) ADP-rhosylation factor-like 6 interacting protein (ARL63P)	AA670422 AA629584 AA464198 AA676460 AA919053 H20652 AA251527 T82415	11 12 11 10 0.9 11	0.9 11 0.7 0.5 0.5 0.5	19 17 20 0.8 12 0.9 0.8	0.8 0.8 11 12 0.8 10 0.9	11 12 11 10 0.9 11	0.3 11 0.7 0.5 0.5 0.5	10 0.5 0.4 0.5 0.3 0.4	11 12 11 10 0.9 11	10 0.9 11 0.9 12 10 0.7	0.8 0.4 0.6 0.7 0.6 0.7	10 0.9 10 12 11 10
Protein Trafficing	ADP-rhosylation factor 4 - Alas (ARF41) ADP-rhosylation factor 3 (ARF3) ADP-rhosylation factor 3 (ARF3) EID-S3DB-aroscitted proteins 5 (EB-AP5) Euryopherin alpha 2 (GPNA2) Euryopherin alpha 2 (GPNA2) ADP-rhosylation factor-18de 6 interacting protein (ARL6P) Luryopherin (mportini) beta 1 (GPNB1) RAB2, nember IKA5 onsognes family (RAB2) mackeopenin SSD0 (AUPIS5)	AA670422 AA629584 AA64198 AA676460 AA919053 1120652 AA251527 T82415 R21785	11 12 11 10 0.9 11 0.9	0.9 11 0.7 0.5 0.5 0.5 0.5	19 17 20 0.8 12 0.9 0.8 0.8 0.8	0.8 0.8 11 12 0.8 10 0.9 0.8	11 12 11 10 0.9 11 0.9	11 0.7 0.5 0.5 0.5 0.5 0.5	10 0.5 0.4 0.5 0.3 0.4 0.3	11 12 11 10 0.9 11 0.9	10 0.9 11 0.9 12 10 0.7 11	0.8 0.4 0.6 0.7 0.6 0.7 0.6	10 0.9 10 12 11 10 0.7
Protein Trafficing	ADP-reboxylation factor 4 - Alas (ARP41) ADP-reboxylation factor 3 (ARP3) ADP-reboxylation factor 3 (ARP3) EB-SSLD-areacostical proteins 6 (EB-AP3) Earyopheria alpha 2 (CPNA1) ADP-reboxylation factor 3 (CB-AP3) ADP-reboxylation factor 3 (AB3) ADP-reboxylation factor 3 (AB3) ADP-reboxylation factor 3 (CB-AP3) ADP-reboxylation particle 7 (AD (SBP72)) Eary Department of the ADS (SBP72)	AA670422 AA629584 AA464198 AA676460 AA919053 H20652 AA251527 T82415 R21785 W32523	11 12 11 10 0.9 11 0.9 0.9	0.9 11 0.7 0.7 0.5 0.5 0.5 0.5 0.6	19 17 20 0.8 12 0.9 0.8 0.8 0.8 0.8	0.8 0.8 11 12 0.8 10 0.9 0.8 108	11 12 11 10 0.9 11 0.9 0.9	11 0.7 0.5 0.5 0.5 0.5 0.5 0.6	10 0.5 0.4 0.5 0.3 0.4 0.3 0.3 0.3	11 12 11 10 0.9 11 0.9 0.9	10 0.9 11 0.9 12 10 0.7 11 0.8	0.8 0.4 0.6 0.7 0.6 0.7 0.6 0.7	10 0.9 10 12 11 10 0.7 11
Protein Trafficing	ADP-rhosylation factor 4 - Alas (ARF41) ADP-rhosylation factor 3 (ARF3) ADP-rhosylation factor 3 (ARF3) EIG-SSDB-arosocited proteins 5 (EB-AP5) Earyopherin alpha 2 (GPNA2) ADP-rhosylation factor - 1 AdA (SPNB3) ADP-rhosylation factor - 1 AdA 6 - unteracting protein (ARL6P) Laryopherin (mportin) beta 1 (GPNB3) Component SPA 6 - Society (ARL82) mechoperin SPA 10 (AUPS) RAB2, member RAS o society (ARL82) mechoperin SPA (UPUPS) signal recognition particle 72LD (SEP72)	AA670422 AA629584 AA64958 AA676460 AA676460 AA676460 AA676460 AA676460 AA6751527 T82415 R21785 W32523	11 12 11 12 11 10 0.9 11 0.9 0.9	0.9 11 0.7 0.5 0.5 0.5 0.5 0.5	19 17 0.8 12 0.9 0.8 0.8 0.8 0.8	0.8 0.8 11 12 0.8 10 0.9 0.8 108	11 12 11 10 0.9 11 0.9 0.9	0.9 11 0.7 0.5 0.5 0.5 0.5 0.5 0.6	10 0.5 0.4 0.3 0.3 0.4 0.3 0.3	11 12 11 10 0.9 11 0.9 0.9	10 0.9 11 0.9 12 10 0.7 11 0.8	0.8 0.4 0.6 0.7 0.6 0.7 0.6 0.7	10 0.9 10 12 11 10 0.7 11
Protein Trafficing	ADP-reboylation factor 4 - Alas (ARP41) ADP-reboylation factor 3 (ARP3) ADP-reboylation factor 3 (ARP3) ADP-reboylation factor 3 (ARP3) Ell-SSDB-attexented protein S (EB-AP5) Euryopherin (importino beta 3 (EB-AP5) Euryopherin (importino beta 3 (EB-AP5) Euryopherin (importino beta 3 (EB-AP5) Euryopherin (importino beta 1 (EB-AB1) Euryopherin (importino beta 1 (EB-AB1) Euryopherin (importino beta 1 (EB-AB1) Euryopherin (importino beta 1 (EB-AB1) Euryopherin (importino beta 1 (EB-AB1)) Euryopherin (importino beta	AA670422 AA629584 AA464958 AA67460 AA570460 AA57052 AA251527 T82415 R21785 W32523 AA630776	13 11 12 11 10 0.9 11 0.9 0.9 0.9	0.9 11 0.7 0.5 0.5 0.5 0.5 0.6	19 17 200 0.8 12 0.9 0.8 0.8 0.8 0.8 0.8 10	0.8 0.8 11 12 0.8 10 0.9 0.8 108 0.9	11 12 11 10 0.9 11 0.9 0.9 0.9	0.9 11 0.7 0.5 0.5 0.5 0.5 0.5 0.6	10 0.5 0.4 0.5 0.3 0.4 0.3 0.3 0.3 10	13 11 12 11 10 0.9 11 0.9 0.9 0.9	10 0.9 11 0.9 12 10 0.7 11 0.8 10	0.8 0.4 0.6 0.7 0.6 0.7 0.6 0.7 1.1	10 0.9 10 12 11 10 0.7 11 0.9
Protein Trafficing	ADP-rhospitation factor 4 - Alas (ARF41) ADP-rhospitation factor 3 (ARF3) ADP-rhospitation factor 3 (ARF3) EID-SDDB-arospitation factor 3 (ARF3) Iaryopherin alpha 2 (GPNA2) Iaryopherin alpha 2 (GPNA2) ADP-rhospitation factor-1846 6 interacting protein (ARL6P) Iaryopherin (mportini) beta 1 (GPNB1) RAB2, nember KA5 onsogene family (RAB2) macheoportin SB20 (OUPIS5) signal recognition particle 724D (SRP72) signal recognition particle 724D (SRP72) signal recognition oparticle 7240 (SRP72) signal recognition particle 724D (SRP72) signal recognition softial 2 (OPS26)	AA670422 AA629384 AA464198 AA676460 AA919033 H20652 AA251527 T82415 R21785 W32523 AA630776 AA604946	11 13 11 12 11 10 0.9 11 0.9 0.9 0.9 0.9	0.9 11 0.7 0.5 0.5 0.5 0.5 0.6	L9 17 5-8 0.8 12 0.9 0.8 0.8 0.8 0.8 0.8 0.8 10 0.8	0.8 0.8 11 12 0.8 10 0.9 0.8 108 0.9 10	11 12 11 10 0.9 11 0.9 0.9 0.9 0.7 10	11 0.7 0.7 0.5 0.5 0.5 0.5 0.6 28 0.5	10 10 0.5 0.4 0.3 0.4 0.3 0.3 10 0.3	13 11 12 11 10 0.9 11 0.9 0.9 0.7 10	10 0.9 11 0.9 12 10 0.7 11 0.8 10 0.9	0.8 0.4 0.6 0.7 0.6 0.7 0.6 0.7 0.6 0.7 L1 0.5	10 0.9 10 12 11 10 0.7 11 0.9 10
Protein Trafficing	ADP-rhospitation factor 4 - Alas (ARP41) ADP-rhospitation factor 3 (ARP43) ADP-rhospitation factor 3 (ARP43) ADP-rhospitation factor 3 (ARP43) Ell-SSDB-artexectided proteins 6 (Ell-AP5) Euryopherin (mpmotils) beta 3 (CPNB3) ADP-rhospitation factor-Bade 6 interacting protein (ARL6 IP) ADP-rhospitation factor-Bade 6 interacting protein (ARL6 IP) RAD2, number 18.45 conceptor Bade (ARL6 IP) RAD2, number 18.45 conceptor Bade (ARL6 IP) RAD2, number 18.45 conceptor Bade (ARL6 IP) and and a statistical and a statistical and (ARL6 IP) RAD2, number 18.45 conceptor Bade (ARL6 IP) and and a statistical and (ARL6 IP) and (ARL6 IP)	AA670422 AA629584 AA664998 AA664998 AA676460 AA990053 H20652 AA251527 T82415 R21785 W32523 AA630776 AA064946 AA97609	11 11 12 11 10 0.9 11 0.9 0.9 0.9 0.7 10 0.9	0.9 11 0.7 0.5 0.5 0.5 0.5 0.5 0.6 20 0.5 0.5 0.5 0.5	L9 17 5 00 0.8 12 0.9 0.8 0.8 0.8 0.8 0.8 0.8 10 0.8	0.8 0.8 11 12 0.8 10 0.9 0.8 108 0.9 10 10	11 12 11 10 0.9 11 0.9 0.9 0.9 0.7 10 0.9	0.9 11 0.7 0.5 0.5 0.5 0.5 0.6 2.8 0.5 0.5 0.6	10 10 0.5 0.4 0.3 0.4 0.3 0.4 0.3 0.3 10 0.3 10 0.3 10	L3 11 12 11 10 0.9 11 0.9 0.9 0.7 10 0.9	10 0.9 11 0.9 12 10 0.7 11 0.8 10 0.9 0.8	0.8 0.4 0.6 0.7 0.6 0.7 0.6 0.7 1.1 0.5 0.7	10 0.9 10 12 11 10 0.7 11 0.9 10 0.9
Protein Trafficing	ADP -rhospitation factor 4 - Alas (ARP41) ADP -rhospitation factor 3 (ARP3) ADP -rhospitation factor 3 (ARP3) EID - SLDD-arrowstated proteins 6 (EID-AP3) EID - SLDD-arrowstated proteins (EID-AP3) EID - rhospitations factor 3 (EID-AP3) EID - rhospitations factor 3 (EID - AP3) EID - related proteins complex 3, defis 1 should (AP3) EID - related proteins complex 3, defis 1 should (AP3) EID - related proteins complex 3, defis 1 should (AP3) EID - related proteins complex 3, defis 1 should (AP3) EID - related proteins complex 3, defis 1 should (AP3) EID - related proteins (EID - AP3) EID - Rhospitations (EID - AP3) EID - Rhospit	AA670422 AA529584 AA664198 AA664198 AA6756460 AA99053 102652 AA251527 T82415 R21785 W312523 AA630776 AA630776 AA630776 AA697609 H22481	11 11 12 11 10 0.9 11 0.9 0.9 0.7 10 0.9 11	0.9 11 0.7 0.5 0.5 0.5 0.5 0.5 0.6 28 0.9 0.4	19 17 200 0.8 12 0.9 0.8 0.8 0.8 0.8 0.8 10 0.8 10 0.8	0.8 0.8 11 12 0.8 10 0.9 0.8 108 0.9 10 10 0.9	11 11 12 11 10 0.9 11 0.9 0.9 0.9 0.9 10 0.9 11	0.9 11 0.7 0.5 0.5 0.5 0.5 0.5 0.6 2.8 0.5 0.5 0.5 0.5 0.5 0.5 0.5 0.5	10 10 0.5 0.4 0.3 0.4 0.3 0.3 10 0.3 10 0.4	L3 11 12 11 10 0.9 11 0.9 0.9 0.7 10 0.9 11	10 0.9 11 0.9 12 10 0.7 11 0.8 10 0.9 0.8 0.9	0.8 0.4 0.6 0.7 0.6 0.7 0.6 0.7 1.1 0.5 0.7 0.7	10 0.9 10 12 11 10 0.7 11 0.9 10 0.9 10
Protein Trafficing	ADP-rhospitation factor 4 - Alas (ARF41) ADP-rhospitation factor 3 (ARF43) ADP-rhospitation factor 3 (ARF43) ADP-rhospitation factor 3 (ARF43) EII-S3DD-areacoristical proteins 5 (EII-AP5) karyopherin (mpmotini) beta 1 (OPMB3) ADP-rhospitation factor-like 6 interacting protein (ARL64P) ADP-rhospitation factor-like 6 interacting protein (ARL64P) RAD2, anesher RAS onso gene family (RAD2) melosports BSD0 (UPUPS) ignal recognition partiale 724D (SRP21) ignal recognition partiale 724D (SRP22) ignal recognition orging 24 (AP324) ignal protein ording 24 (AP324) ignal recognition ording 24 (AP326) ignal recognition ording 24 (AA670422 AA22954 AA264198 AA76460 AA97053 100652 AA251527 T82415 R21785 W32523 AA630776 AA004946 AA97059 1122481 189170	11 13 11 12 11 10 0.9 11 0.9 0.9 0.7 10 0.9 11 10 10 11 10 11 10 10 11 10 10	10 0.9 11 0.7 0.5 0.5 0.5 0.5 0.6 28 0.5 0.9 0.4	19 17 20 0.8 12 0.9 0.8 0.8 0.8 0.8 0.8 10 0.8 10 0.8	0.8 0.8 11 12 0.8 10 0.9 0.8 108 0.9 10 10 0.9 0.8	13 11 12 11 10 0.9 0.9 11 0.9 0.9 0.9 0.7 10 0.9 11 10	0.9 11 0.7 0.7 0.5 0.5 0.5 0.6 2.8 0.5 0.9 0.4 2.1	10 10 0.5 0.4 0.3 0.4 0.3 0.4 0.3 10 0.3 10 0.3 10 0.3 10 0.3 10 0.3 10 0.3 10 0.3 10 0.4 10 0.4 10 0.4 10 0.4 10 0.4 10 0.4 10 0.4 10 0.4 10 0.4 10 0.4 10 0.4 10 0.4 10 0.4 10 0.4 10 0.4 10 0.5 10 10 10 10 10 10 10 10 10 10	L3 L1 L2 L1 L0 0.9 L1 0.9 0.9 0.7 10 0.9 L1 10 0.9 L1 10 0.9 10 10 10 10 10 10 10 10 10 10	10 0.9 11 0.9 12 10 0.7 11 0.8 10 0.9 0.8 0.9	0.8 0.4 0.6 0.7 0.6 0.7 0.6 0.7 1.1 0.5 0.7 0.7 0.7	10 0.9 10 12 11 10 0.7 11 0.9 10 0.9 10 0.8
Protein Trafficing	ADP-rhospitaio factor 4 - Alas (ARP41) ADP-rhospitaio factor 3 (ARP3) ADP-rhospitaio factor 3 (ARP3) EB-SSD2-arcsociated protes 5 (EB-AP3) karyophera alpha 2 (CPNA2) Maryophera alpha 2 (CPNA2) Maryophera (mportia) beta 1 (CPNB1) ADP-rhospitaio factor-site 6 intercenting protein (ARL6 P) karyophera (mportia) beta 1 (CPNB1) RAD2, mether PLAS occopece fmolty (RAL2) methoportia ISE0 (NUPS5) signal recognition particle 7230 (SEP72) adaptor-related protein complex 3, della 1 submit (AP3D1) vacuular protein sorting 24 (VP226) warman protein sorting 12 (VP226) Cabricular (NPTK) della Complexation (SEC6) methoportia (NPTK) della Complexation (SEC6) methoportia (NPTK) della Complexation (SEC6) methoportia (NPTK) della Complexation (SEC6) Cabricular precursor	AA670422 AA2954 AA464198 AA664198 AA67640 AA97053 1120652 AA25527 T82415 K21785 W33523 AA630756 AA61946 AA61946 AA71669 H22481 H99170	11 13 11 12 11 10 0.9 11 0.9 0.9 0.7 10 0.9 11 10 0.9 11 10 0.9 11 10 0.9 11 10 0.9 11 10 10 10 10 10 10 10 10 10	0.9 11 0.7 0.5 0.5 0.5 0.6 24 0.5 0.9 0.4 21	19 17 20 0.8 12 0.9 0.8 0.8 0.8 0.8 0.8 0.8 0.8 10 0.8 10 0.8 10 22 10	0.8 0.8 11 12 0.8 10 0.9 0.8 108 0.9 10 10 0.9 0.8	13 11 12 11 10 0.9 0.9 0.9 0.9 0.7 10 0.9 11 10	0.9 11 0.7 0.7 0.5 0.5 0.5 0.5 0.5 0.5 0.5 0.5	10 10 0.5 0.4 0.5 0.3 0.4 0.3 0.3 10 0.3 10 0.4 17	L3 L1 L2 L1 L0 0.9 L1 0.9 0.9 0.7 10 0.9 L1 10 0.9 L1 10 0.9 10 0.9 11 10 0.9 10 10 10 10 10 10 10 10 10 10	10 0.9 11 0.9 12 10 0.7 11 0.8 10 0.9 0.8 0.9 16	0.8 0.4 0.6 0.7 0.6 0.7 0.6 0.7 0.7 0.7 0.7 0.7 0.7 0.5	10 0.9 10 12 11 10 0.7 11 0.9 10 0.9 10 0.8
Protein Trafficing	ADP-rhospitation factor 4 - Alas (ARP41) ADP-rhospitation factor 3 (ARP43) ADP-rhospitation factor 3 (ARP43) ADP-rhospitation factor 3 (ARP43) EID-SDDA-associated proteins 5 (EB-AP5) karyopherin (mpmotini) beta 1 (GVRB3) ADP-rhospitation factor-like 6 unteracting protein (ARL6P) ADP-rhospitation factor-like 6 unteracting protein (ARL6P) Karyopherin (mpmotini) beta 1 (GVRB3) ADP-rhospitation factor-like 6 unteracting protein (ARL6P) karyopherin (mpmotini) beta 1 (GVRB3) RAB2, number RAS ensogene family (ARL2) melooportin SSQ (URDES) argunt recognition particle 72kD (SRP72) argunt recognition particle 72kD (SRP72) structure protein or ring 22 (VPR26) trummal postraxia (NPTX) Cabericului precursor Protein fibospitatas 2, resultatory submit B, (PPP991)	AA670422 AA62954 AA64195 AA64953 H00532 H00532 AA640532 H2145 H2145 H2145 H2275 W32323 AA630756 AA64946 AA71609 H02441 H99170 H5154	11 13 11 12 11 10 0.9 11 0.9 0.9 0.9 0.7 10 0.9 11 10 10 10 10 10 10 10 10 10	10 0.9 11 0.7 0.5 0.5 0.5 0.5 0.5 0.5 0.5 0.6 2.4 0.9 0.4 2.1 15	19 17 50 0.8 12 0.9 0.8 0.8 0.8 0.8 10 0.8 10 10 3.2	0.8 0.8 1.1 12 0.8 10 0.9 0.8 100 10 0.9 10 10 0.9 0.8	13 11 12 11 10 0.9 11 0.9 0.9 0.9 0.9 0.9 11 10 0.9 11 10	0.9 11 0.7 0.7 0.5 0.5 0.5 0.5 0.5 0.5 0.5 0.5	10 10 0.5 0.4 0.5 0.3 0.3 0.4 0.3 0.3 10 0.3 10 0.4 17 14	L3 L1 L2 L1 L0 0.9 L1 0.9 0.7 10 0.9 L1 10 0.9 11 10 10	10 0.9 11 0.9 12 10 0.7 11 0.8 10 0.9 0.8 0.9 16	0.8 0.4 0.6 0.7 0.6 0.7 0.6 0.7 0.7 0.5 0.7 0.7 0.7 0.5	10 0.9 10 12 11 10 0.7 11 0.9 10 0.9 10 0.8
Protein Trafficing	ADP-rhospitation factor 4 - Alas (ARP41) ADP-rhospitation factor 3 (ARP43) ADP-rhospitation factor 3 (ARP43) ADP-rhospitation factor 3 (ARP43) Ele -SSDB-artexostical proteins 6 (Ell=AP5) Euryphenis (migmetinis beta 3 (CBPA3) Euryphenis (migmetinis beta 3 (CBPA3) Euryphenis (migmetinis beta 3 (CBPA3) Euryphenis (migmetinis beta 1 (CBP3) Euryphenis (migmetinis beta 1 (CBP3)) Euryphenis (CBP3) Euryphenis (CBP3) Euryphe	AA670422 AA2954 AA464198 AA7640 AA97053 1120652 AA25527 T82415 R21785 W33523 AA630756 AA630756 AA630756 H212481 H99170 H61869 H61869 H618785	11 11 12 11 10 0.9 0.9 0.9 0.9 0.7 10 0.9 11 10 10 0.9	10 0.9 11 0.7 0.5 0.5 0.5 0.5 0.5 0.5 0.5 0.5 0.5 0.5	19 17 5-0 0.8 12 0.9 0.8 0.8 0.8 0.8 10 0.8 10 0.8 10 0.8 10 10 2.2 10	0.8 0.8 1.1 1.2 0.8 1.0 0.9 0.8 1.08 0.9 1.0 1.0 0.9 0.8 0.8 1.0	13 11 12 10 0.9 11 0.9 0.9 0.9 0.9 0.9 0.9 0.9 0.9 11 10 10	0.9 11 0.7 0.7 0.5 0.5 0.5 0.5 0.6 2.8 0.5 0.9 0.4 2.1 19 10.	10 0.5 0.4 0.3 0.3 0.4 0.3 0.3 10 0.3 10 0.4 17 14	L3 L1 L2 L1 L0 0.9 L1 0.9 0.7 10 0.9 L1 10 0.9 L1 10 0.9 11 10 0.9 11 10 0.9 11 10 0.9 11 0.9 0.9 11 0.9 0.9 11 0.9 0.9 11 0.9 0.9 0.9 0.9 11 0.9 0.9 0.9 0.9 11 0.9 0.9 0.9 0.9 0.9 0.9 0.9 0.9	10 0.9 11 0.9 12 10 0.7 11 0.8 10 0.9 0.8 0.9 16 11	0.8 0.4 0.6 0.7 0.6 0.7 0.6 0.7 1 1 0.5 0.7 0.7 0.7 0.7 0.5	10 0.9 10 12 11 10 0.7 11 0.9 10 0.9 10 0.8 10 0.8
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atornet Protein Trafficing	ADP-rhospitaion factor 4 - Alas (ARP41) ADP-rhospitaion factor 3 (ARP43) ADP-rhospitaion factor 3 (ARP43) ADP-rhospitaion factor 3 (ARP43) EB-53DD-attexent deprotes 15 (EB-AP5) karyopherin (mpmotils) beta 3 (EB-AP5) ADP-rhospitaion factor-1646 & interacting protein (ARL6 IP) ADP-rhospitaion factor-1648 & interacting protein (ARL6 IP) ADP-rhospitaion factor-1648 & interacting protein (ARL6 IP) anticoportis ISBD (AUDPS) ignal racognition particle 7210 (SRP2) inflator-testical protein complex 3 defia 1 submati (AP3D1) vacualar protein sorting 26 (VP326) imitor 16 Screenwise Seedon and is nortegicisus rece6 (SEC6) merusing leastance (ARL7 N) Cabricular protein complex (AXL3) protein phosphatase 2, regulatory submit B (PPP28.3) AXL receptor (tyrosine kanase (AXL3)	AA670422 AA2954 AA64198 AA7640 AA797053 II20652 AA25527 T82415 R2185 W3253 AA630776 AA630776 AA630776 II22481 II99170 N63865 II5738 II5738	11 12 11 12 11 10 0.9 0.9 0.9 0.9 11 10 0.9 11 10 0.9 11 10 0.9 11 10 10 0.9 11 10 11 10 11 11 12 11 11 12 11 11 12 11 15 11 15 15 15 15 15 15 15	10 0.9 11 0.7 0.5 0.5 0.5 0.5 0.6 24 0.5 0.9 0.4 21 10 10 10 10 10 10 10 10 10 1	19 17 0.8 12 0.8 0.8 0.8 0.8 0.8 0.8 0.8 0.8 0.8 0.8	0.8 0.8 11 12 0.8 10 0.9 0.8 100 10 0.9 10 10 0.9 0.8 10 0.8 10 0.8	11 11 12 11 10 0.9 0.9 0.9 0.9 0.9 0.9 0.9 10 0.9 11 10 0.9 11 10	0.9 11 0.7 0.5 0.5 0.5 0.5 0.5 0.5 0.5 0.5	10 10 0.5 0.4 0.3 0.3 0.4 0.3 10 0.3 10 0.4 17 14 13 20 0.2	L3 L1 L2 L1 L0 0.9 L1 0.9 0.7 10 0.9 L1 10 0.9 L1 10 0.9 L1 10 0.9 L1 10 0.9 L1 10 0.9 11 10 0.9 11 10 0.9 11 10 0.9 11 10 0.9 11 10 0.9 11 10 0.9 11 10 0.9 11 10 0.9 11 10 0.9 11 10 0.9 11 10 0.9 11 10 0.9 11 10 0.9 11 10 0.9 10 10 0.9 11 10 0.9 10 10 0.9 11 10 0.9 11 10 0.9 11 10 0.9 11 10 0.9 11 10 0.9 11 10 0.9 11 10 0.9 11 10 0.9 11 10 0.9 11 10 0.9 11 10 0.9 11 10 0.9 11 10 0.9 11 10 10 10 10 10 10 10 10 10	10 0.9 11 0.9 12 10 0.7 11 0.8 10 0.9 0.8 0.9 16 11 10 13 10 0.9	0.8 0.4 0.6 0.7 0.6 0.7 0.6 0.7 0.7 0.7 0.7 0.7 0.7 0.5 0.9 0.8 0.8 0.8	10 0.9 10 12 11 10 0.7 11 0.9 10 0.9 10 0.8 10 0.9

Figure 9.

Transcript levels of genes encoding DNA repair, molecular chaperones, antioxidant proteins, ribosomal proteins and elongation factors, proteasomes, and proteinases and their inhibitors, as well as genes responsible for protein trafficking, sorting, secretion, and quality control functions subcategories and other genes (PP2A, AXL, B4GALT1, and MVK) are regulated by OS. Upregulated, downregulated, and unchanged genes are shown in *red, green*, and *yellow*, respectively. Genes considered unchanged genes were those with ratios 1 ± 0.2 ; upregulated genes were those with ratios between 1.2 and 2 (*light red*) and above 2 (*dark red*); downregulated genes were those with ratios between 0.5 and 0.8 (*light green*) and less than 0.5 (*dark green*).

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Primer sets and Reaction Conditions

Gene Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	PCR Product (bp)	Annealing Temperature (° C)
Copper zinc superoxide dismutase (CuZnSOD)	GGGGAAGCATTAAAGGACTGACT	AATTACACCACAAGCCAAACGAC	353	52
Glutamylcysteine synthetase (GCSH) Glutathione S-transferase (GSTT2)	TGAGGCCAACATGCGAAAAC CCAGCCGCGCGTCTACATCT	GGCTTGGAATGTCACCTGGAG AGTCGTGGCCGTCCCTCAAACAG	409 532	55 61
Manganese superoxide dismutase (Mn SOD)	CTCCCGACCTGCCCTACGACTAC	AAACCAAGCCAACCCCAACCTGAG	374	60
Thioltransferase (TTase)	CTGGGAAGGTGGTTGTGTT	TTCCTATGAGATCTGTGGTTACTG	305	47
Glyceraldehyde-3-	CCACCCATGGCAAATTCCATGGCA	TCTAGACGGCAGGTCAGGTCCACC	600	60
CD 47	ACGTAAGGGTCTCATAGGT	CTCTTCGGATTTCAGGTTT	254	44
Amyloid precursor protein (APP)	CCACTGCAGGCACGTTGTA	CCGCCTGGCCCCTGGAGAA	260	58
<i>β</i> -Tubulin	GGGAAGGGGACCATGTTGACTG	CTGGGACCATGGACTCTGTTCG	573	56
Mitogen-activated kinase interacting protein 2	CAGGCCTGAAGGATGATGGT	AGTGGCAAATGCAGGAGAGG	296	52
N-acetyllactosamine synthase (B4GLT1)	GCCACTGAATGCGAGAAACC	CGGGAAAATTGGGAGAGAGTGCT	289	56
Fanconi anemia group A gene	CCAAGGCCATGTCCGACTCG	CAGAAAGCATGGCCTGGCGACG	581	60
Catalase	TACCCTCCTGGACTITTTACATC	CCTCATTCAGCACGTTCACATAGA	541	52