The oxidized thiol proteome in aging and cataractous mouse and human lens revealed by ICAT labeling

Benlian Wang, Grant Hom, Sheng Zhou, Minfei Guo, Binbin Li, Jing Yang, Vincent M. Monnier, and Xingjun Fan

1Center for Proteomics, Case Western Reserve University, Cleveland, OH 44120, USA
2Department of Pathology, Case Western Reserve University, Cleveland, OH 44120, USA
3State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, China
4Department of Ophthalmology, The Huichang County People’s Hospital, Jiangxi, China
5Department of Ophthalmology, Ganzhou City People’s Hospital, Jiangxi, China
6Department of Biochemistry, Case Western Reserve University, Cleveland, OH 44120, USA

Summary

Age-related cataractogenesis is associated with disulfide-linked high molecular weight (HMW) crystallin aggregates. We recently found that the lens crystallin disulfide bond distribution and distribution and reproduction in any medium, provided the original work is properly cited.

Introduction

The mammalian genome encodes 214 000 cysteine residues (Go & Jones, 2013). Some of the cysteine residue pairs are playing a key role in high-ordered protein structure and conformation through intradisulfide bonds (Borges & Sherma, 2014). These cysteine pairs tend to be evolutionarily conserved (Thornton, 1981), and any disturbance of this precise disulfide bond formation will likely have a detrimental effect on protein function. Many of the free cysteine residues are also actively involved in redox signaling. It has been estimated that 10–20% of cysteine residues are redox-active (Go & Jones, 2013). These cysteine residues, together with other redox regulatory components such as glutathione (GSH), oxidized glutathione (GSSG), cysteine, thioredoxin (Trx), and thioredoxin reductase (TrxR), constitute a powerful force in maintaining intracellular and extracellular redox status. Oxidation of these cysteine residues will give rise to an imbalance between intra- or extracellular redox state, which may trigger regulatory gene expression and alter protein stability (Furukawa et al., 2006). Finally, a large number of cysteine residues are not directly involved in protein folding and redox signaling, but can have a profound impact on protein conformation and function when becoming oxidized (Gross et al., 1996). Over the years, several groundbreaking methods and techniques have offered researchers great opportunities to systematically study protein cysteine oxidation, including cysteine sulfenylation, sulfenylation, sulfonylation, nitrosylation, and disulfide formation (Paulsen & Carroll, 2013).

The present study constitutes a logical extension of a recent study in which we have systematically mapped the ‘disulfidome’ of crystallins from the aging and cataractous human lens and found surprisingly conserved oxidation sites between human age-related cataracts, the LEGSKO mouse model of age-related cataract linked to glutathione depletion, and oxidative changes in mouse lens homogenate exposed to H2O2 in vitro (Fan et al., 2015). In a nutshell, cysteine oxidation into disulfides was highly conserved in the β-crystallin family, but only partly conserved in the γ-crystallin family, implying complex in vivo unfolding mechanisms underlying the oxidation of sites not readily accessible to H2O2 and other cellular oxidants. While these results brought invaluable information for the potential links between de novo intra- and intermolecular disulfide formation and impaired crystallin structure and assembly, they did not provide information on the quantitatively minor but functionally critical noncrystallin proteins involved in controlling the redox state, overall metabolism, and cytoskeleton functions of the lens.

Previous studies by others have shed partial light on the presence and potential roles of noncrystallin oxidation sites in human and rodent models of cataracts. Padaonkar et al. (1999) reported a significant loss of lens cytoskeletal protein in guinea pigs after hyperbaric oxygen (HBO) treatment and proposed that the loss of thiol group was due to disulfide metabolism or cytoskeletal architecture, strongly suggesting that they have a pathogenic role in cataractogenesis. By extrapolation, these findings may be of broader significance for age- and disease-related dysfunctions associated with oxidant stress. Key words: aging; cataractogenesis; disulfide; mass spectrometry; proteomics; reactive oxygen species.
cross-linking. In another study (Padgaonkar et al., 1989), they found that more than 95% of glyceroldehyde-3 phosphate dehydrogenase (GAPDH) activity was depleted in rabbit lens after 24-h HBO treatment. In a later study, Yan et al. (2006) found GAPDH activity in aged and cataractous lens can be recovered by thioredoxin (Trx) and thioredoxin reductase (TrxR) further supporting the notion that disulfide bond formation may be responsible for the inactivation of the enzyme. Disulfide exchange has been indicated as playing a key role in aldose reductase activity, and the formation of mixed disulfides has also been found in the lens (Mizoguchi et al., 1999). Lou MF et al. (Lou & Dickerson, 1992) have reported that protein-thiol mixed disulfides generally increase with aging in the human lens.

While each of these studies provided valuable information, then available techniques did not allow researcher to systematically identify and quantitate abnormal cysteine disulfide-forming target proteins. ICAT is a newly developed approach to tackle this problem. ICAT reagent contains a biotinylated tag with high selectivity and reversible affinity (Shiio & Aebersold, 2006). Using affinity purification through avidin-agarose beads, one can enrich only the peptides with disulfide bond. This greatly simplifies probing the huge pool of a mixture of peptides from lens digest. More importantly, the relative extent of cysteine oxidation can be easily estimated by simple comparison of the integrated peak intensities in the doublets corresponding to the heavy and the light ICAT labels. For this reason, we mined the ICAT labeling data that we recently deposited into the ProteomeXchange Consortium, for the purpose of identifying (i) all noncrystallin disulfides that are common to human and LEGSKO mouse cataracts and in their in vitro oxidation, (ii) the sites and patterns of these cysteine residues that are oxidized during aging and oxidation, and (iii) the potential impact on lens biology based on their molecular functions. By mapping the cysteine residues oxidation sites and patterns in vitro and in vivo, we hope to better understand the significance of the sulfhydryl groups in lens biology as they have also been linked to various diseases other than cataractogenesis (Grek & Townsend, 2014).

Below we report that mining the oxidative proteome of the human and LEGSKO mouse cataractous lens has helped us to uncover and identify extensive disulfide formation sites in cysteine residues that are critical for the proper function of key cellular proteins involved in lens metabolism, such as GAPDH, redox homeostasis, such as peroxiredoxin 6 (PRDX6), and cytoskeletal structure, such as phakinin (BFSP2) and filensin (BFSP1).

**Results**

Three sets of data were collected in this study as illustrated in Fig. 1. They constitute a map of the cysteine oxidation sites detected in aged normal and aged cataractous human lens, GSH-deficient mouse (LEGSKO mouse) lens, and the in vitro modeling of the mouse lens protein extract oxidized with 1 and 5 mM hydrogen peroxide (H2O2) for up to 12 h. It is important to understand that each peptide reported below represents the ratio of the peptide labeled with the heavy chain (C13 ICAT stable isotope) that is present in either aged normal human lens, aged cataractous human lens, LEGSKO or hydrogen peroxide-oxidized mouse lens protein extract, vs. the identical peptide labeled with the light chain (C12 ICAT isotope) present in extract from young human lens, age-matched WT mouse lens, and mouse lens extract without hydrogen peroxide oxidation. Therefore, the ratio of ICAT heavy/ICAT light reflects the relative degree of disulfide bonding occurring in the aged normal and aged cataractous human lens peptides vs. the degree of disulfide bonding occurring in the same peptides from young control lenses. The same principle applies to LEGSKO vs. age-matched control and H2O2-oxidized vs. nonoxidized control. The concept of how ICAT labeling and dimethyl labeling are used to quantitatively determine disulfide bond is illustrated in Fig. S1 (Supporting information), and the typical mass spectrum of ICAT and dimethyl labeling is shown in Figs S2 and S3 (Supporting information). All the MS data, excluding crystallins, were quantitatively analyzed after adjustment.

**Human Lens**

| Normal aged lens nucleus | Normal young nucleus pool | Type II-V cataractous nucleus | LEGSKO cataractous lens | Age-matched WT lens |
|--------------------------|--------------------------|-----------------------------|-----------------------|-------------------|
| C13 ICAT labeling        | C13 ICAT labeling        | C13 ICAT labeling           | C13 ICAT labeling     | C13 ICAT labeling |

**Mouse Lens**

| 3-month-old mouse lens protein extract |
|----------------------------------------|
| 1 mM H2O2 2 hr                        |
| 0 mM H2O2 2 hr                        |
| 5 mM H2O2 12 hr                       |
| 1 mM H2O2 12 hr                       |
| 0 mM H2O2 12 hr                       |
| 5 mM H2O2 12 hr                       |

**In vitro modeling**

| C12 ICAT labeling | C13 ICAT labeling | C12 ICAT labeling | C13 ICAT labeling |
|-------------------|-------------------|-------------------|-------------------|
| 1:1               | 1:1               | 1:1               | 1:1               |
| T2B               | T2C               | T12B              | T12C              |

**Digestion, purification, affinity enrichment, MS analysis, quantification based on dimethyl labeling, excluding crystallin proteins**

**Fig. 1** Summary of the experimental design and analytical procedures that are used in this study. For in vitro modeling experiment, the sample identifications (T2B, T2C, T12B, and T12C) are listed under each oxidation condition.
with dimethyl labeling to compare levels of the individual protein. We only report those proteins that are both involved in disulfide formation and for which both deuterated and nondeuterated dimethyl-labeled peptides could be identified based on Mascot ion score 20 and above. The identified dimethyl-labeled peptides are listed in Tables S2 and S3 (Supporting information). Finally, we tested three samples in each sample set, and only the peptides detected in all three samples are reported in this study.

A similar pattern of proteins involved in disulfide bond formation is found in human and mouse cataract and in vitro modeling by oxidation with H\textsubscript{2}O\textsubscript{2}.

Seventy-four oxidized proteins in either aged normal or aged cataractous human lenses (see Table 1), 50 proteins in either LEGSKO or WT mouse lenses (see Table 2), and 54 proteins in either nonoxidized or in vitro-oxidized mouse lens protein extract (see Table S1, Supporting information) were detected by ICAT proteomic analysis. These were categorized based on their molecular functions using the Panther classification system (www.pantherdb.org). As illustrated in Fig. 2A–C, 46.7%, 38.0%, and 44.9% of disulfide-forming proteins that were identified in human lens, mouse lens, and in vitro oxidation, respectively, are those with catalytic activity, such as the enzymes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sorbitol dehydrogenase (SORD), and others. The second most prevalent group includes structural proteins, with 24.0%, 26.0%, and 30.6% of proteins identified in human, mouse lenses, and in vitro modeling experiments, respectively. These include several structural proteins unique to the lens, such as beaded filament structural protein 1 and 2 (BFSP1 and BFSP2). We also found a large number of binding proteins involved in disulfide bond formation, which accounted for 22.7%, 22.0%, and 18.4% in human, mouse, and in vitro modeling of the proteins, respectively. We also found proteins with enzyme regulator activity, nucleic acid binding transcription factor, receptor activity, transporter activity, transporter regulatory activity, and antioxidant activity-related functions (see Fig. 2A–C, and Tables 1 and 2 and S1, Supporting information). Overall, of 50–74 proteins, 17 were found in all three sets of pools (see Table 3), and 23 proteins shared the same identity in human vs. mouse lens in vivo, while 23 proteins shared the same identity in human lens vs. in vitro data, and 26 proteins shared the same identity between mouse in vivo and in vitro modeling (Fig. 2D).

Significant cysteine residue oxidation occurs in lens intermediate filament and other structural proteins

Intermediate filaments (IFs) are playing a critical role in cell cytoskeleton integrity (Song et al., 2009). The cysteine residue 167 located in the filensin (BFSP1) central rod domain was oxidized into disulfide at moderate levels in aged human lenses, while significantly oxidized in category II to V cataractous lenses (Fig. 3A). The mouse filensin Cys290 with the same motif as Cys167 in the human was also found to be eightfold oxidized compared to age-matched WT lenses (Fig. 3B). Interestingly, this was also reproduced by in vitro oxidation with H\textsubscript{2}O\textsubscript{2} (Fig. 3B; also see Table S1, Supporting information). Similarly, the Cys65 located in the head domain of phakinin (BFSP2) was not detectable in aged normal and grade II cataractous human lenses, while intensively oxidized at grade III to V cataractous human lenses (Fig. 3C). The oxidation and disulfide formation at Cys326 located at the rod domain of phakinin was mild in old normal and category II cataractous human lenses but significantly oxidized in higher-grade cataractous lenses (Fig. 3C). However, we did not detect phakinin disulfide cross-linking from mouse and in vitro modeling samples. We also detected a large number of cysteine residues forming disulfide bond in cytokeratin proteins, particular in human lenses. This included Cys51, Cys77, and Cys474 in keratin 6A; Cys42 and Cys489 in keratin 2; Cys25, Cys66, and Cys427 in keratin 10; Cys55 and Cys474 in keratin 5; Cys432 in keratin 9; Cys40, Cys18, and Cys389 in keratin 14; and Cys49 and Cys497 in keratin 1 (Table 1). The majority of these cysteine residue oxidation sites were positively associated with degrees of cataract severity. Some of the cysteine residues in keratin 2 and keratin 14 were completely oxidized in grade V cataractous lenses (ratio >1000; Table 1). Although keratin oxidation, especially cysteine oxidation, has not been reported in the lens, several studies have implicated disulfide bonds in regulating the cytoskeletal assembly and organization in skin keratinocytes (Feng & Coulombe, 2015). This includes Cys40 in the head domain of keratin 14, which we also detected in the lens proteins.

Several IF-associated structural proteins were also identified in our study, including lengsin, vimentin, and plectin. The oxidation of Cys1026 in plectin was not detectable in old normal human lenses, while found to be completely oxidized in grade II to V cataractous human lenses. Similarly, the Cys170 in lengsin was detected in grade III and above cataractous human lenses (Table 1). The Cys326 in vimentin was 26 times oxidized in LEGSKO vs. age-matched WT mice lenses (Table 2). In addition, we also found microfilaments and microtubule proteins that were involved in disulfide cross-linking, such as actins and tubulins. These cytoskeleton proteins are important components in lens cell structure and organization. It has been shown that disrupting actin assembly by UV light or cytochalasin D promotes cataracts in rat lenses (Mousa et al., 1979), and interferes with tubulin disulfide bond formation by selenium. This was proposed as a mechanism in cataract formation in the selenium cataractogenesis animal model (Leynadier et al., 1991). As illustrated in Fig. 3D, Cys285 motif in gamma- and beta-actin (ACTG1, ACTB, and ACTBL2) was not detected in aged normal and grade II cataractous human lenses, while found significantly oxidized in grade III to V cataractous human lenses. The Cys217 in gamma- and beta-actin (ACTG1 and ACTB) was mildly oxidized in aged normal human lenses, but significantly oxidized in cataractous lenses and associated with severity of cataractous grade. Similar results were seen in LEGSKO lens vs. age-matched WT lenses and in vitro modeling of H\textsubscript{2}O\textsubscript{2} oxidized lens protein extract vs. nonoxidized controls (Fig. 3E).

We found twofold elevation of the Cys347 in tubulin in LEGSKO lenses compared to age-matched WT lenses, but no tubulin proteins were detected in human lens samples (Tables 1 and 2). The lens capsule component proteins, such as various type IV collagen chains, were mostly detected in mouse lenses and in vitro oxidation. This might be due to the fact that the entire mouse lens was used in our study, while only the lens nucleus was used in the human studies. Strikingly, several cysteine residues in type IV collagen were dramatically oxidized in the LEGSKO compared to WT lenses (Fig. 3F), indicating that oxidation affects not only the lens body but also the lens capsule during aging.

Disulfide cross-linking affects a large number of enzymes

As we have stated above, a large number of enzymes were identified, including hydrodase, deaminase, small GTPase, nucleotide phosphatase, isomerase, peptidase, ligase, oxygenase, dehydrogenase, cysteine protease, kinase, and reductase. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was well detected in all three sets of sample pools. The Cys247 located in the catalytic domain of GAPDH in human lens and its similar motif Cys271 in mouse lens were found involving in disulfide bond formation. The oxidation of Cys247 in human lenses was not
Table 1 Relative amount of disulfide bonding in peptides from proteins of aged normal and cataractous human lenses versus young lenses

| Protein name | Description                                      | Peptide Sequence†‡ | ICAT<sub>Young</sub> or ICAT<sub>Young</sub>§‡§‡§‡§ |
|--------------|--------------------------------------------------|--------------------|-----------------------------------------------|
|              |                                                  |                    | Aged Normal | Type II cataract | Type III cataract | Type IV cataract | Type V cataract |
| Antioxidant Activity |                                              |                    |              |                 |                 |                 |                 |
| PRDX6        | Peroxiredoxin 6                                 | DFTPVC47TELGR      | 0.03 (± 0.003) | 0.02 (± 0.001) | 0.02 (± 0.001) | 0.025 (± 0.001) | 0.01 (± 0.001) |
| Calcium Binding |                                              |                    |              |                 |                 |                 |                 |
| DSG1         | Desmoglein-1                                    | IHSDC77AAQQVTTYR   | 0.64 (± 0.07) | 3.02 (± 0.39)  | 5.88 (± 0.29)  | 18.29 (± 1.11) | 34.28 (± 5.67) |
| S100A7L2     | Protein S100-A7-Like 2                          | ENFPLFLGC58EK      | ND           | ND               | ND               | 12.23 (± 1.91) | 25.51 (± 0.98) |
| S100A7A      |                                                  | QSHGAPC965GSGQ     | 0.45 (± 0.11) | 0.66 (± 0.09)  | 0.89 (± 0.21)  | 0.55 (± 0.08)  | 0.67 (± 0.12)  |
| S100A8       |                                                  | LLET4EC6PYR       | ND           | 0.69 (± 0.06)  | 1.04 (± 0.08)  | 1.89 (± 0.22)  | 2.22 (± 0.72)  |
| Catolytic Activity |                                              |                    |              |                 |                 |                 |                 |
| AMDHD1       | Imidazolonepropionase-related                    | C305SAILPTTAYMLR   | ND           | 18.90 (± 2.38) | 41.12 (± 6.01) | 85.4 (± 10.98) | >1000           |
| CDA          | Cytidine deaminase                              | SAYC31FHSHP/VAGALTQEGR | ND | 17.18 (± 2.21) | 54.34 (± 9.11) | 123.45 (13.31) | 112.35 (21.11) |
| FAH          | Fumarylacetoacetase                             | IGFGQC408AGK       | ND           | 2.62 (± 0.12)  | 5.70 (± 0.31)  | 6.02 (± 0.31)  | 8.05 (± 1.21)  |
| CDC42        | Cell division control protein 42 homolog        | YVEC157SALTOK      | ND           | ND               | >1000           | >1000           | >1000           |
| RHOG         | Pho-related GTP-binding protein Rhog            | C6VVG6GDGAVGK      | ND           | 1.83 (± 0.31)  | 2.34 (± 0.73)  | 6.88 (± 1.11)  | 12.78 (± 1.37) |
| RAC1         | Ras-related C3 butylin toxin substrate 1        | YLEC157SALTQR      | ND           | ND               | ND               | ND               | 56.818181828    |
| Mfn2         | Mitofusin 2                                     | C188PLK            | ND           | ND               | ND               | 25.25 (± 5.62) | >1000           |
| UCHL1        | Ubiquitin C-terminal hydrolase L1               | FSAVALC220K        | 13.56 (± 1.19) | 20.79 (± 1.02) | 17.45 (± 4.15) | 56.17 (± 8.98) | 98.03 (± 9.72) |
| SERPINB6     | Serpin peptidase inhibitor, clade B (ovalbumin), member 6 | TNGILFC374GR  | ND           | 5.77 (± 0.26)  | 12.43 (± 0.74) | 20.04 (± 2.17) | 71.94 (± 7.29) |
| SERPINB9     | Serpin peptidase inhibitor, clade B (ovalbumin), member 9 | ANSLFC370GR  | 6.28 (± 0.34) | 20.04 (± 4.27) | 19.72 (± 3.12) | 43.29 (± 7.98) | 71.94 (± 5.39) |
| ABCA10       | ATP binding cassette subfamily A member 10      | C679SDQGIR         | ND           | ND               | ND               | 121.9512195    | >1000           |
| TR1          | Triosephosphate isomerase 1                     | IYYGGSVATGC218K    | ND           | 23.41 (± 1.04) | 29.78 (± 2.29) | 47.32 (± 9.81) | 70.92 (± 7.74) |
| LGSN         | Lengsin, lens protein with glutamine synthetase domain | VC170D7FTVGPLLTSPR | ND           | ND               | 22.83 (± 0.81) | 129.8 (± 4.19) | >1000           |
| GSS          | Glutathione synthetase                          | C294DIA1TQLATGKT   | 2.17 (± 0.36) | 77.57 (± 11.38) | >1000           | >1000           | >1000           |
| ENO1         | Enolase 1                                       | FGANA1LGSVALC26K   | 0.12 (± 0.02) | 0.78 (± 0.06)  | 1.04 (± 0.02)  | 1.12 (± 0.27)  | 1.33 (± 0.19)  |
| SORD         | Sorbitol dehydrogenase                          | VLVCL179GAPGIGMVTLLVAK | ND | 109 (± 7.3)   | >1000           | >1000           | >1000           |
| ALDH1A1      | Aldehyde dehydrogenase                          | IGPALS186GNTVVKPAEQTPTALHVASL1K | ND | 3.9 (± 0.98) | 22.02 (± 3.29) | 13.71 (± 7.72) | 15.33 (± 2.78) |
| GMPR         | Guanosine monophosphate reductase               | STC316TVGAAK       | ND           | 3.16 (± 0.33)  | 5.54 (± 0.72)  | 6.18 (± 1.17)  | 15.62 (± 4.01) |
| CBR1         | Carbonyl reductase 1                            | SC150SPELQQK       | 39.37 (± 2.21) | 69.44 (± 11.16) | 41.15 (± 8.89) | 64.93 (± 13.37) | 23.14 (± 5.53) |

(Continued)
Table 1 (Continued)

| Protein name | Description | Peptide Sequence†‡ | ICATNormal/ICATYoung or ICATcat/ICATYoung§§ | Aged Normal | Type II cataract | Type III cataract | Type IV cataract | Type V cataract |
|--------------|-------------|-------------------|---------------------------------------------|-------------|----------------|----------------|----------------|----------------|
| GAPDH        | Glyceraldehyde-3-phosphate dehydrogenase | VPTANV5VWDLTC247R | ND          | 23.25 (+ 0.78) | 44.24 (+ 3.46) | 73.52 (+ 8.02) | >1000         |
| HPD          | 4-hydroxyhexylpyruvate dioxygenase | QAASFVC375 | 2.48 (+ 0.17) | 18.34 (+ 1.74) | 13.47 (+ 2.87) | 11.33 (+ 2.44) | 65.35 (+ 8.83) |
| LDHA         | Lactate dehydrogenase | VIGGGG105NDSAR | ND          | 181.26 (+ 23.16) | 112.15 (+ 17.27) | >1000         |
| MDH1         | Malate dehydrogenase 1 | AIC269DHR | >1000       | >1000           | >1000           | >1000           |
| PRDX6        | Peroxiredoxin 6 | DFTPVC4T7TELGR | 0.01 (+ 0.003) | 0.02 (+ 0.001) | 0.03 (+ 0.001) | 0.025 (+ 0.001) | 0.02 (+ 0.001) |
| TKT          | Transketolase | TVFC386STFAAFFTR | ND          | 476.19 (+ 19.78) | >1000           | >1000           |
| AKR1B1       | Aldo-keto reductase family 1, member B1 (aldo reductase) | LIQCYC200OSK | ND          | ND              | ND              | ND              | 312.5 (+ 22.43) |
| NME1         | NME1NM23 nucleoside diphosphate dehydrogenase 1 | GDFC109QVGR | ND          | ND              | ND              | ND              | 16.55 (+ 1.33) |
| PGK1         | Phosphoglycerate kinase 1 | AC108ANPAAGS VILNE | ND          | 131.57 (+ 9.72) | >1000           | >1000           |
| PCMT1        | Protein-L-isoaspartate (O-aspartate) O-methyltransferase | ALDVGGSGLTCAC153FAR | ND          | 102.04 (+ 11.21) | >1000           | >1000           |
| IPOS         | Importin 5 | TIECS7B0ILAVGK | ND          | 22.37 (+ 2.22) | >1000           | >1000           |
| PARK7        | Parkinsonism-associated deglycase | DVPQOC46SR | 4.87 (+ 0.78) | 30.48 (+ 2.28) | 34.60 (+ 4.21) | 70.92 (+ 7.84) | >1000         |
| RNF149       | Ring finger protein 149 | GCC118TDFK | ND          | >1000           | >1000           | >1000           |
| Enzyme regulator protein | | | | | | | |
| PGCL1        | Phospholipase C gamma 1 | SSLRLEPC1088AISIEVLR | ND          | 38.75 (+ 4.42) | 41.15 (+ 2.87) | 48.78 (+ 7.73) | 64.93 (+ 5.41) |
| Nucelic acid binding transcription factor (PARK7 can also be categorized in this section) | | | | | | | |
| WIZ          | Widely interspaced zinc finger motifs | C1456FTGNS RAYVQHA KLM | ND          | 13.19 (+ 1.02) | >1000           | >1000           |
| Receptor Activity | | | | | | | |
| LSAMP        | Limbic system-associated membrane protein | VDVYDEGSYTC111SVQTH EPK | 0.07 | 0.04 | 0.14 | 0.27 | 0.98 (+ 0.03) |
| PLA2R1       | Phospholipase A2 receptor 1 | CS1QAGK | 1.35 (+ 0.04) | 1.77 (+ 0.26) | 7.56 (+ 1.17) | 21.42 (+ 7.52) | 27.08 (+ 9.13) |
| COL4A1       | Collagen type IV alpha 1 | SAFDEC1616HR | 0.24 | 8.13 (+ 1.12) | 34.28 (+ 5.24) | 63.79 (+ 13.21) | >1000         |
| Structure (COL4A1 can also be categorized in this section) | | | | | | | |
| FBVI         | Fibrillin 1 | C1171VNLIGK | 37.91 (+ 5.17) | 234.67 (+ 30.14) | >1000           | >1000           |
| KRT6A        | Keratin 6A | GSGGGGSCA51GAGGGSR | 1.23 (+ 0.21) | 7.86 (+ 0.93) | 12.56 (+ 1.73) | 42.55 (+ 9.21) | >1000         |
| LLEGEC47AR  | | | | | | | |
| BFSP1        | Beaded filament structural protein 1 | SSYDC167R | 5.9 (+ 0.13) | 46.94 (+ 2.05) | 39.21 (+ 1.74) | 57.47 (+ 4.03) | 101.01 (+ 8.02) |
| ACTG1/ACTB  | Actin gamma 1, actin, beta | C285DVDIR | 4.02 (+ 0.47) | 24.81 (+ 2.33) | 53.47 (+ 3.92) | 47.28 (+ 11.43) | 119.04 (+ 14.01) |
| ACT82       | Actin, beta-like 2 | LC217TVYALDFQEMATAASSSLEK | ND          | ND              | 21.27 (+ 1.11) | 32.01 (+ 6.02) | 39.19 (+ 4.91) |
| KRT2        | Keratin 2 | STSFSSC42LSR | 0.83 (+ 0.05) | 3.40 (+ 0.43) | 14.90 (+ 3.01) | 102.48 (+ 9.02) | >1000         |
| KRT10       | Keratin 10 | GSSGGGCG66F6GSSGYYGLGF GGGF | 0.49 | 0.30 | 1.03 (+ 0.08) | 3.56 (+ 0.13) | 8.78 (+ 1.03) |

(Continued)
Table 1 (Continued)

| Protein name | Description | Peptide Sequence | ICATaged/ICATYoung or ICATcat/ICATYoung |
|--------------|-------------|-----------------|-----------------------------------------|
|              |             |                 | Aged Normal | Type II cataract | Type III cataract | Type IV cataract | Type V cataract |
| KRT5         | Keratin 5   | AETEC427QYVQOLLIK | 2.27        | 7.71 (± 1.03)    | 16.12 (± 3.25)   | 18.19 (± 6.03)   |
| KRT9         | Keratin 9   | VSLAGAC55GVGAGYSSR | 6.82 (± 0.84) | 10.03 (± 1.27)  | 29.58 (± 1.71)   | 96.15 (± 10.03)  |
| KRT14        | Keratin 14  | LLEGEC474R       | 2.26 (±0.72) | 37.04 (± 7.99)   | 428.11 (± 21.05) | >1000            |
| KRT1         | Keratin 1   | QBEIC432QOEEYSSLR | ND          | 2.02 (± 0.33)    | 2.58 (± 0.49)    | 5.17 (± 0.81)    |
| BFSP2        | Beaded filament structural protein 2, phakinin | GSC18BIGGIGGSSR | 3.37 (± 0.21)  | 7.92 (± 1.03)    | 19.84 (± 3.41)   | >1000            |
| PLEC         | Plectin     | ALGEC497APNVSVST | ND          | 21.00 (± 4.02)   | 77.13 (± 10.10)  | >1000            |
| SPAN1        | Spectrin alpha, nonerythrocytic 1 | C389EMEQQNQEYK | 3.98 (± 0.45)  | 14.90 (± 2.05)   | 120.48 (± 13.48) | >1000            |
| DSP          | Desmoplakin | NQIC1206EQPQVQER | 0.53 (± 0.02) | 14.78 (± 2.37)   | 372.5 (± 21.02)  | >1000            |
| PRC1         | Protein regulator of cytokinesis 1 | QLEIC1026ETR | ND          | >1000            | >1000            | >1000            |
| LIM2         | Lens intrinsic membrane protein 2 | YC4BLGNK | 0.28        | 0.62 (± 0.02)    | 0.73 (± 0.02)    | 0.20 (± 0.01)    |
| SCNN1B       | Sodium channel epithelial 1 beta subunit | IIIC4EGPKKK | ND          | >1000            | >1000            | >1000            |
| RNF149       | Ring finger protein 149 | GGC118TFKDK | ND          | ND              | ND              | 434.13 (± 24.89) |
| FABP5        | Fatty acid binding protein 5 | TQTF5C67TGLGEK | 12.21 (± 0.17) | 15.24 (± 0.09)   | 27.88 (± 1.05)   | 38.75 (± 1.37)   | 47.84 (± 4.92)   |
| ALDOA        | Aldolase, fructose-bisphosphate A | RC02QVYTEK | 7.71 (± 1.12)  | 27.47 (± 3.26)   | 32.57 (± 2.87)   | 54.43 (± 8.01)   | 48.54 (± 7.23)   |
| MIF          | Macrophage migration inhibitory factor | YAS1C7BOQNGPSKEILPDGDHDUK | 3.99 (± 0.23)  | 29.67 (± 0.72)   | >1000            | >1000            |
| ALB          | Albumin     | YIC289EQLQSSSK | 0.04        | 0.15            | 0.76             | 0.92 (± 0.03)    | 6.79 (± 0.37)    |
| BCO1        | $\beta$C16 corepressor-like 1 | C1121GKEK | ND          | ND              | ND              | >1000            |
| FND3A        | Fibronectin type III domain containing 3A | ALV1C189K | 1.03        | 1.05 (± 0.02)    | 1.01 (± 0.01)    | 1.92 (± 0.22)    | 4.21 (± 0.37)    |
| PRDM1        | PR domain 1 | NELC239PK | ND          | ND              | ND              | >1000            |
| YWHAZ        | Tyrosine 3-monooxygenase | DSGC49YLSLLEK | 6.94 (± 0.74) | 44.89 (± 2.38)   | >1000            | >1000            |
| RBP1         | Retinol binding protein 1 | VEGLVC198K | ND          | >1000            | >1000            | >1000            |
| PKM          | Pyruvate kinase | GIPVLC548K | 2.8 (± 0.03) | 12.2 (± 0.28)    | 54.94 (± 7.12)   | 91.34 (± 10.89)  | 77.1 (± 13.54)   |
| PRDM1        | PR domain 1 | NELC239PK | ND          | ND              | ND              | >1000            |
non-crystallin disulfidome of the lens, B. Wang (Continued)

| Protein name | Description | Peptide Sequence | Value | Value | Value |
|--------------|-------------|-----------------|-------|-------|-------|
| HBB          | Hemoglobin subunit beta | AGKPVIC400ATQMLESMIK | ND    | ND    | ND    |
| MFAP2        | Microfibrillar-associated protein | EEQYPC103TR | 0.32  | 0.21  | 0.42  |
| PEP1         | Phosphatidylethanolamine binding protein 1 | IHC112LENVDK | ND    | 1.26  | 0.11  |
| SPTBN1       | Spectrin beta, nonerythrocytic 1 | IHC112LENVDK | ND    | 1.26  | 0.11  |
| MFAP2        | Microfibrillar-associated protein | EEQYPC103TR | 0.32  | 0.21  | 0.42  |
| PEP1         | Phosphatidylethanolamine binding protein 1 | IHC112LENVDK | ND    | 1.26  | 0.11  |
| SPTBN1       | Spectrin beta, nonerythrocytic 1 | IHC112LENVDK | ND    | 1.26  | 0.11  |

- The ratio of disulfides in normal aged and cataractous lens vs. young normal control was determined as ICAT-13C/ICAT-12C.
- The peptides from crystallins were excluded.
- The ICAT ratio has been adjusted based on dimethyl labeling results.
- ND means not detectable.
- Value < 1 indicates the less disulfide bond formation in aged normal and aged cataractous lenses compared to control; value > 1000 indicates the significant abundant peak identified in MS is by heavy ICAT label.

- The amino acid sequence number of the cysteine residues was assigned by counting the N-terminal methionine as residue 1.
- The ICAT also has been adjusted based on a dimethylation labeling result.
- The value of standard error (SE).
- Value > 1000 indicates the significant abundant peak identified in MS is by heavy ICAT label.

Disulfide cross-linking affects antioxidative defense proteins

Aldehyde dehydrogenase is considered as a lens antioxidative protein as described above. We have also found that other proteins involved in disulfide bond formation may have a significant impact on lens antioxidative status, such as glutathione synthase (GSS) and peroxiredoxin (PRDX6). GSS is one of the glutathione de novo synthesis enzymes. We found that its 294th cysteine residue (Cys225 in mouse) was moderately oxidized in aged normal human lenses and significantly oxidized in grade II cataractous human lenses, while drastically (ratio > 1000) oxidized in grade III and above cataractous human lenses.
as illustrated in Fig. 5A. Strikingly, this cysteine residue (Cys225) was also strongly oxidized in LEGSKO vs. WT mice lenses (Fig. 5A). However, we did not find modifications of GSS during in vitro oxidation. As for PRDX6, we surprisingly found much higher (30- to 100-fold) oxidation/disulfide bond formation at Cys47 in young human lenses compared to aged normal and cataractous human lenses as indicated in Fig. 5B. However, no oxidation was detected in the LEGSKO/WT mouse lens or in the in vitro-oxidized samples. The oxidative stress sensor, Parkinsonism-associated deglycase (DJ-1/PARK7), was also detected in all our samples. As illustrated in Fig. 6A,B, Cys46 and Cys53 were oxidized in aged normal and aged cataractous human lenses. Both cysteine residues were also found significantly oxidized in LEGSKO lenses and in vitro oxidation by H\textsubscript{2}O\textsubscript{2} when compared to their controls.

**Discussion**

The above data represent to our knowledge the first comprehensive and comparative survey of noncrystallin disulfide bonds forming in age-related human cataract and a mouse model of low antioxidant defense (the LEGSKO mouse) similar to that found in the nuclear region of the human lens. A major strength of this study is that many of the oxidation sites could be modeled in vitro by oxidation with H\textsubscript{2}O\textsubscript{2} and can be related to the lens crystallin oxidation sites recently published by us (Fan et al., 2015). The pending question however is: What is the meaning of these findings and how do the detected oxidation sites relate to the work by others? In particular, the ICAT method used in this work does not allow us to attribute the observed disulfides to protein–protein or mixed disulfides (PSSG and PSSC), with, for example, glutathione and cysteine, which have been well documented to increase in aged human lenses (Lou, 2003). In addition, while the method is powerful for comparison of relative changes in oxidized proteins, it is limited in that it does not provide information on the percentage of oxidation at a particular site. Thus, additional studies for each of the main targets identified will be needed to understand the physiological meaning of the findings.

Of importance, however, is that the majority of proteins involved in disulfide cross-linking are those with enzymatic functions, which constitute at least one-third of the proteins identified in this study. The key question is whether the cysteine residue(s) oxidation affects the enzymatic activity of the protein. Indeed, the aging lens has been associated with a declining activity in several enzymes, such as lactate dehydrogenase (LDH) (Zhu et al., 2010), ubiquitin conjugation enzymes (Shang et al., 1997), and thiol repairing enzymes (Wei et al., 2015), such as glutathione reductase (GR), thioredoxin reductase (TR), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

**GAPDH oxidation**

GAPDH is known to be prone to inactivation by oxidant stress. It has been extensively studied in the lens and found to be inactivated in aging and cataractous lens, and also by hyperbaric oxygen (HBO) and photoxidation (Padgaonkar et al., 1989; Xing & Lou, 2002). It plays an enormous role in controlling glycolysis rate and thus cellular homeostasis as various lens ATP-dependent biological functions are relying on ATP production by GAPDH. Yan et al. (2006) found thioredoxin (Trx) and thioredoxin reductase (TrXR) were able to revive inactivated GAPDH in aged normal and cataractous human lenses. These results clearly indicate that disulfide formation must be in part the trigger causing protein conformational change or blocking the cysteine site in or close to the active site. The impact of weakened GAPDH activity might also be responsible for the accumulation of methylglyoxal-derived advanced glycation end products, as proposed by Brownlee (Brownlee, 2001). This may result in a paradoxical improvement of chaperone activity of α-crystallin (Kanade et al., 2009), while methylglyoxal itself can cause mitochondrial dysfunction, ER activation, and lens epithelial cell apoptosis (Palsamy et al., 2014).

**Aldehyde dehydrogenase oxidation**

Another metabolic enzyme hit by oxidation was aldehyde dehydrogenase. In the eye, this enzyme is described as both ‘corneal and lens crystallin’ (Marchitti et al., 2011). In addition, Lassen et al. (2007) have reported that ALDH1A1 may also have an antioxidative function, that is, prevent UV-induced damage. In the same study, ALDH1A1 knockout mice were found developing cataracts at 6–9 months of age especially sensitive to UV light. Interestingly, the mechanism of inhibition of aldehyde dehydrogenase by disulfiram, a drug used to treat chronic alcoholism, was found actually to involve the disulfide formation (Vallari & Pietruszko, 1982).

**Oxidation of redox enzymes**

Equally important are those enzymes involved in controlling the redox state of the lens such as PRDX6 and GSS. The Cys294 was highly oxidized in cataractous human lenses, and a similar result was also found in LEGSKO mice lenses (Cys225), although the GSS peptides found from in vitro modeling did not make the Mascot score threshold possibly due to loss in the insoluble fraction. In addition, due to either low abundance or mass spectrometry sensitivity, a large number of proteins that were identified by ICAT could not be quantitatively determined due to lack of dimethyl-labeled peptides. This may also explain why we did not report some of mitochondrial electron transporter chain-related enzymes, which have been reported prone to oxidation (Musatov & Robinson, 2012; Wu et al., 2014). On the other hand, it has also been reported (Xing & Lou, 2010) that some of the lenticular enzymes are more resistant to oxidative stress, such as thioredoxin reductase and glutathione reductase. This might also explain why some key redox enzymes were missing from this study. Future work is needed to verify whether disulfide bond formation by the cysteine residue may affect GSH de novo synthesis.

Quite in contrast to GSS, we found 30 to 100 times less disulfide bond formation at Cys47 in PRDX6 in aged normal and aged cataractous human lenses compared to the young lens pool. The peroxidase active site of PRDX6 motif is PVC47TT. The Cys47 is actively engaged in photooxidation (Padgaonkar et al., 1989; Xing & Lou, 2002). It plays an enormous role in controlling glycolysis rate and thus cellular homeostasis as various lens ATP-dependent biological functions are relying on ATP production by GAPDH. Yan et al. (2006) found thioredoxin (Trx) and thioredoxin reductase (TrXR) were able to revive inactivated GAPDH in aged normal and cataractous human lenses. These results clearly indicate that disulfide formation must be in part the trigger causing protein conformational change or blocking the cysteine site in or close to the active site. The impact of weakened GAPDH activity might also be responsible for the accumulation of methylglyoxal-derived advanced glycation end products, as proposed by Brownlee (Brownlee, 2001). This may result in a paradoxical improvement of chaperone activity of α-crystallin (Kanade et al., 2009), while methylglyoxal itself can cause mitochondrial dysfunction, ER activation, and lens epithelial cell apoptosis (Palsamy et al., 2014).

**Oxidation of cytoskeleton proteins**

Major oxidative changes were also observed in cytoskeleton proteins. It is well documented that these proteins play a critical role in lens transparency (Quinlan et al., 1999). In our study, all three major cytoskeleton proteins, microfilaments, microtubules, and intermediate...
| Protein name | Description | Sequence†,‡ | ICATLEGSKO/ICATWT§,¶,††,‡‡ | Homologous motif found in human |
|-------------|-------------|-------------|-------------------------|-------------------------------|
| **Binding** | Fibulin 1 | DC50SLPYTSESK | 64.10 (± 8.18) | |
| | | LGES234NTGVSFR | 25.06 (± 4.69) | |
| | Parkinsonism-associated degylcase | DPVQC46SR | 13.93 (± 2.17) | Cys46 |
| | Eukaryotic translation initiation factor 4A1 | AILPC66IK | 14.95 (± 1.82) | |
| | Spectrin alpha, nonerythrocytic 1 | GAC1627AGSEDAVK | 68.51 (± 11.03) | Cys157 |
| | Ras-related C3 botulinum toxin substrate 1 | YLEC157SALTQR | 14.26 (± 3.17) | |
| | Importin 5 | TIEC560ISGLAVGK | >1000 | Cys578 |
| | Eukaryotic translation elongation factor 2 | YVPEVDVPC4662NVGLGVQDLVFLVK | 11.96 (± 1.15) | |
| | | STLTDSSLDC41K | 4.78 (± 0.78) | |
| | | TFC290QLDFRRK | 18.80 (± 3.55) | |
| | Clusterin | EGEDDRTVC284K | >1000 | |
| | MLX interacting protein | TSSC118HLSIDASLTK | 521.21 (± 21.19) | |
| | Fibrillin 1 | C1173VNUGK | >1000 | |
| | | NGE2420VNDR | 625.00 (± 70.36) | |
| | | C2232PVGYVLR | >1000 | |
| | | C1960NEGVEAPDGR | 6.24 (± 0.44) | |
| | Latent transforming growth factor beta binding protein 2 | | |
| **Catalytic activity** (PARK7, EIF4A1, RAC1, IPO5, and EEF2 can also be categorized in this section) | Cathepsin B | SC211EAGYSPSYK | 34.25 (± 3.01) | |
| | Triosephosphate isomerase 1 | IAVAAOQC117YK | >1000 | |
| | | RYGGSVTGAC268K | 12.38 (± 2.22) | Cys218 |
| | | C225PDIAIQLAGTK | >1000 | Cys294 |
| | HtrA serine peptidase 1 | TYTNLC130QLR | 5.30 (± 1.17) | |
| | Enolase 3 | TGAPC399R | 7.93 (± 0.31) | |
| | | VNIQGVTEIAC357K | 6.47 (± 1.00) | |
| | Glyceraldehyde-3-phosphate dehydrogenase | AICAG485GK | 40.03 (± 7.38) | |
| | | VTPFNC386STFAAFFTR | 6.76 (± 0.83) | Cys386 |
| | HECT domain and ankyrin repeat containing E3 ubiquitin protein ligase 1 | | |
| | | GANPNYQDISGC99TPLHLAAR | 3.09 (± 0.12) | |
| | Phosphoglycerate dehydrogenase | VNVC234AR | 9.43 (± 0.88) | |
| | | ALVDHENVSC2B1PHLGASTK | 3.11 (± 0.15) | |
| | | ALQSGQC254AAGAALDFEPPRDR | 4.3 (± 0.50) | |
| | Argininosuccinate synthase 1 | FELTC132YSLAPQIK | 9.92 (± 2.06) | |
| | | YLTLGSLARPC97IAL | 17.51 (± 9.91) | |
| | Hengsin | TNMF3C385SGSVR | 62.72 (± 7.25) | |
| | | VIC224DTFTGEPILPSR | 8.27 (± 1.11) | Cys170 |
| | | DLKDSVPTTWGYNDNSC445ALNLK | 3.08 (± 0.39) | |
| | | ITSC63FQLQEAGIK | 5.38 (± 0.20) | |
| | Phosphoribosylaminoimidazole carboxylase | | |
| | | IGPAISC187GNTVVKPAEQTPLTLAHLSLKI | 21.94 (± 1.17) | Cys186 |
| | | VFNAYLSDLGCG126IK | 22.36 (± 7.33) | Cys126 |
| | | LECD370GGRG | 22.83 (± 3.06) | |
| | Ubiquitin C-terminal hydrolase L1 | FSAIVALC220K | 12.29 (± 1.04) | Cys220 |
| | | NEAQAAHDSVQSEG GC152R | 4.18 (± 0.37) | Cys152 |

Enzyme regulator activity (IPO5 and EEF2 can also be categorized in this section)

**Nucleic acid binding transcription factor activity (PARK7 and MIXIP)**

**Receptor activity (COL4A1 can also be categorized in this section)**

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| Protein name | Description | Sequence†‡§¶††‡‡ | ICATLEGSKO/ICATWT§¶††‡‡ | Homologous motif found in human |
|--------------|-------------|-------------------|-----------------------------|--------------------------------|
| COL4A4      | Structure molecular activity (Col4a1, Sptan1, Col4a2, Fbn1, and Ltpb2 can also be categorized in this section) | SQSVRPGADVTFIC1792TAK AAPFVEC1626QGR | 454.55 (± 21.37) 12.95 (± 0.28) | |
| VIM         | Vimentin    | QVQSLMEQNC28QVDALK | 26.2 (± 3.41) | |
| KRT14       | Keratin 14  | C334MEMNOQQEYK    | 1.88 (± 0.10) | Cys389 |
| COL4A2      | Collagen, type IV, alpha 2 | GTC1660HYFANK ATPFIEC1653NGGR AHNQDGLAGSC1532LAR HSQTDQPMCS1499PVGMNK | 146.48 (± 11.04) >1000 362.33 (± 34.04) 635.14 (± 37.92) | |
| ACTBL2      | Actin, beta-like 2 | C285DVDIR | 14.7 (± 2.03) | Cys285 |
| COL4A4      | Collagen type IV alpha 4 | AAPFVEC1626QGR | 12.95 (± 0.93) | |
| TUBA1A      | Tubulin alpha 1a | TIQVDWC347PTGFK C285DVDIR | 1.78 (± 0.08) 14.7 (± 0.62) | Cys285 |
| ACTB        | Actin, beta | LC217YVALDFEQQEDEATGKF YC46LGNK | 8.92 (± 2.07) 1.83 (± 0.12) | Cys48 |
| LIM2        | Lens intrinsic membrane protein 2 | YC46LGNK | 8.48 (± 1.14) | Cys167 |
| Bfsp1       | Beaded filament structural protein 1 | SSYDC290R | 8.48 (± 1.14) | |
|             |              |                  |                | |
|             | Translation regulator activity (EIf4A1 and EEF2 can also be categorized in this section) |                  |                | |
| ATP1B3      | ATPase Na+/K+ transporting subunit beta 3 | IIDLPDGYPQISC191LPK | 4.66 (± 0.46) | |
|             | Other functions |                  |                | |
| FABP5       | Fatty acid binding protein 5 | TTVFSC67NLGKEK TETVCB77FQDGALVQWQVDGK | 6.72 (± 0.72) 6.23 (± 0.25) | Cys67 |
| MIF         | Macrophage migration inhibitory factor | LLCB1GLAER | 31.17 (± 2.14) | Cys81 |
| ALB         | Albumin      | Raelakymc289nqatisk C511fslfvetvypk | 88.13 (± 7.13) 167.03 (± 9.73) | Cys289 |
|             |              | LC99APNLRC TNC416DLYEK DTC591FSTEGNLVTR | 88.49 (± 5.29) 105.26 (± 15.32) 22.46 (± 3.61) | |
|             |              | VC485LHEK | >1000 | |
| PKM         | Pyruvate kinase | GIPVLC474K | 13.86 (± 0.88) | Cys548 |
|             |              | NTGRC49TIGPASR AGKPVICS26ATQMLESMIK | 19.76 (± 2.04) 2.64 (± 0.09) | Cys123 |
| MFAP2       | Microfibrillar-associated protein 2 | TVC144AHEELLR | 125.00 (± 8.32) | |
| PFN1        | Profilin 1   | C128YEMASHLR | 45.73 (± 3.83) | |
| VTN         | Vitronectin  | INC2140GK C287QFGFASK | 233.23 (± 17.17) 4.88 (± 0.67) | |
| ND1         | Nidogen 1    | C837MPGEVSK | 6.68 (± 0.33) | |
| NDUA11      | NADH dehydrogenase | EKPPDPITYFAGGCG97AGGLTLGAR | 36.90 (± 2.18) | |
| CCT4        | Chaperonin containing TCP1 subunit 4 | IGLIQFC252LSAPK | 9.53 (± 1.03) | |
| IDH1        | Isocitrate dehydrogenase 1 | C73ATITPDEK | 0.009 (± 0.001) | |
| HS90AB1     | Heat-shock protein 90 kDa alpha family class B member 1 | FENLC564K | 106.38 (± 13.06) | |

†The amino acid sequence number of the cysteine residues was assigned by counting the N-terminal methionine as residue 1.
‡The peptides from crystallins were excluded.
§This ratio of disulfides in 9-month-old LEGSKO and age-matched wild-type whole lenses was determined as ICAT-13C/ICAT-12C.
¶The ICAT ratio has been adjusted based on dimethyl labeling results.
††The number in parenthesis is the value of standard error (SE).
‡‡Value <1 indicates the less disulfide bond formation in aged normal and aged cataractous lenses compared to control; value >1000 indicates the significant abundant peak identified in MS is by heavy ICAT label.
filaments were found heavily involved in disulfide bond formation in aged, GSH-deficient, and cataractous lenses. IFs are particularly important for lens structure due to the lens' unique high order of cell-cell organization, unusually high protein content (reach to 400 mg mL\(^{-1}\) in the lens nucleus region). Lens-specific IF proteins, beaded filament structural proteins 1 and 2 (BFSP1 BFSP2), also known as filensin and phakinin, through their interactions with chaperones, guide the lens cellular organization and alignment to achieve its high refractive index. The importance of BFSP1 and BFSP2 was further evidenced by their mutation-associated cataract phenotype (Conley et al., 2000). Besides these, other lens structure component proteins were also oxidized, including fibrillin-1 (FBN1), plectin (PLEC), spectrin alpha (SPTAN1), and lens intrinsic membrane protein 2 (LIM2). FBN1 is an important component of lens zonules, and mutation of FBN1 has been found to be associated with ectopia lentis (Khan et al., 2014). Plectin has been found associated with intermediate filament proteins (Wiche & Winter, 2011), and LIM2 is an eye-specific protein found at the junctions of lens fiber cells. However, whether the oxidation and disulfide bond formation in these cysteine residues will cause protein conformational change and ultimately alter lens cytoskeleton organization and aggregate formation remains to be clarified.

**Oxidation of other vital proteins**

Finally, one interesting question is the extent to which the protein cysteine oxidation data obtained in lens are applicable to other cellular systems. Clearly, lens proteins are long-lived proteins and therefore prone to accumulation of damage. Neurons, on the other hand, are postmitotic cells prone to neurodegeneration and similar accumulation of protein damage. In that regard, Parkinsonism-associated deglycase (DJ-1/PARK7) that was detected in all our samples is not only an oxidative stress sensor (van der Merwe et al., 2015), but also a chaperone that inhibits protein

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| Protein Name | Description | Human peptide sequence | LEGSKO mouse peptide sequence | Mouse in vitro peptide sequence |
|--------------|-------------|------------------------|------------------------------|--------------------------------|
| KRT6A        | Keratin 6A  | GSGGLGGAC51GGAGFGSR    | –                            | –                              |
|              |             | ISIGGGSC77AGGYYGSR     | –                            | –                              |
|              |             | LLEGGEC474R            | –                            | –                              |
| BFSP1        | Beaded filament structural protein 1 | SSYDC167R | SSYDC290R | –                              |
| SPTAN1       | Spectrin alpha, nonerythrocytic 1 | ALC315AEADR | – | – |
| AKR1B1       | Aldo-keto reductase family 1, member B1 (aldo dehydrogenase) | LIOYC200QSK | GAC1627AGSEDAVK | GAC1627AGSEDAVK |
| LM2          | Lens intrinsic membrane protein 2 | YC4B87NGK | YC4B67NGK | – |
| UCHL1        | Ubiquitin C-terminal hydrolase L1 | FSAVALC220K | FSAVALC220K | – |
| TR1          | Triosephosphate isomerase 1 | IYYGGSVTGATC218K | IYYGGSVTGATC268K | IAVAAQNC117YK |
| KRT1         | Keratin 1   | FSGC49GGGGSFGAGGGFGSR  | –                            | –                              |
| MFAP2        | Microfibrillar-associated protein 2 | EEQYPCT03TR | – | – |
| ACTG1/ACTB   | Actin gamma 1 | C285VDVDIR | C285VDVDIR | C163VDVDIR |
| RAC1         | Ras-related C3 botulinum toxin substrate 1 | YLEC157ALTQR | YLEC157ALTQR | YLEC157ALTQR |
| LGSN         | Lengsin, lens protein with glutamine synthetase domain | VIC170DFTVTGEPILLTSPR | VIC224DFTVTGEPILLTSPR | VIC224DFTVTGEPILLTSPR |
| ACTBL2       | Actin, beta-like 2 | C286VDVDIR | C286VDVDIR | C286VDVDIR |
| PKM          | Pyruvate kinase | GTPVLC548K | GTPVLC474K | GTPVLC474K |
|            |              | NTGIC123TGIPASR         | NTGIC49TIGIPASR              | NTGIC49TIGIPASR              |
| PRA          | Peptidylprolyl isomerase A | GIDC52FHR | – | – |
| FBN1         | Fibrillin 1  | C1171VNLJIK            | C1173VNLJIK                  | C1173VNLJIK                  |
| ALDH1A1      | Aldehyde dehydrogenase 1 family member A1 | IGPA11S186GNTVVKPAEQTPTTLAHLSUK | IGPA11S187GNTVVKPAEQTPTTLAHLSUK | IGPA11S187GNTVVKPAEQTPTTLAHLSUK |
| ENO1         | Enolase 1    | FEGANILGVSLAVC26K      | VFNAYLSDLCGCC126K            | VFNAYLSDLCGCC126K            |
| KRT5         | Keratin 5    | VSLAGAC55GSGGYYGSR     | –                            | –                              |

(Continued)
| Protein Name | Description | Human peptide sequence | LEGSKO mouse peptide sequence | Mouse in vitro peptide sequence |
|--------------|-------------|-------------------------|-----------------------------|--------------------------------|
| TKT          | Transketolase | LLEGEEC474R              | LLEGEEC473R                  |                                |
| IPO5         | Importin 5   | TIEC5785ILQAVK           | TIEC5605ILQAVK               |                                |
| FABP5        | Fatty acid binding protein 5 | TQFSC67TLGK             | TVFSC67NLGK                  |                                |
| GAPDH        | Glyceraldehyde-3-phosphate dehydrogenase | VPTAN5VVDLT205R          | VPTPN5VVDLT271R               |                                |
| PARK7        | Parkinonism-associated deglycase | DPVQC465R              | AAIC485GK                    |                                |
| COL4A1       | Collagen type IV alpha 1 | SAPFEC1616HGK           | SAPFEC1616HGK                |                                |
| GSS          | Glutathione synthetase | C294PDATQLAGTK          | C225PDIAQLAGTK               |                                |
| ALB          | Albumin      | YIC289ENGQD5SSK          | Raelakymc289e nqattissk       |                                |
| MIF          | Macrophage migration inhibitory factor | LLC81GLLAER            | LLC81GLLAER                  |                                |
| ALDOA        | Aldolase, fructose-bisphosphate A | RC202QYVTEK             | ALANSLAC393QGK               |                                |

Table 3 (Continued)
aggregation, such as α-synuclein (Shendelman et al., 2004). PARK7 has also been reported highly expressed in lens fibers (Sun et al., 2015). There are three cysteine residues in PARK7, Cys46, Cys53, and Cys106. The Cys106 is a critical PARK7 antioxidant mediator. Under oxidative stress, Cys106 will be sulfonated or sulfonated to shift to a more acidic isoform triggering an oxidation elimination response. The Cys46 and Cys53 have

![Fig. 3](image-url)

Representative proteins and their cysteine residues that are involved in disulfide cross-linking in lens structural proteins. (A) The disulfide bond ratio of the Cys167 in filensin (BFSP1) in human lens. (B) The disulfide bond ratio of the Cys290 in mouse BFSP1 in both LEGSKO mouse and in vitro oxidation by H2O2 compared to their control. (C) The disulfide bond ratio of the Cys65 in human phakinin (BFSP2). (D) The Cys217 in various actins (ACTB, ACTG1, and ACTBL2) and the Cys285 in various actins (ACTB and ACTG1) in aged normal and cataractous human lenses compared to young normal control. (E) The actin cysteine residue Cys285 and Cys217 in both LEGSKO mouse and in vitro oxidation by H2O2 compared to their control. (F) Cys1493 and Cys1616 in collagen 4a1, Cys1419, Cys1532, Cys1660, and Cys1653 in collagen 4a2, and Cys1626 in collagen 4a4 were oxidized in LEGSKO vs. age-matched WT mice lenses. Cat-II: grade II cataract; Cat-III: grade III cataract; Cat-IV: grade IV cataract; Cat-V: grade V cataract. T2B, T2C, T12B, and T12C are in vitro oxidation sample pairs (see Fig. 1). All ICAT data are expressed as the ratio vs. young lenses pool, LEGSKO vs. age-matched WT, or oxidized vs. nonoxidized lens protein extract. Standard errors were calculated from three biological replicates. One-way ANOVA was used to compare the significance between groups, and $P < 0.05$ was considered significant. The significance level was marked by either *, **, *** or lower case letters and the significance value was shown inside the figures.
much less regulatory activity than Cys106 (Meulener et al., 2005), but Cys53 was found to be playing an important role in the stabilization of PARK7, so the Cys106 can form heterodisulfide protein complexes (Fernandez-Caggiano et al., 2016). We did not detect Cys106 disulfide in PARK7, but dramatic oxidation was found in Cys46 and Cys53 implying that Cys106 may have been hyperoxidized due to PARK7 protein conformational change via extensive oxidation of Cys46 and Cys53 residues.

In summary, in agreement with the outcome of the crystallin protein cysteine residue oxidation studies in the same lens extracts (Fan et al., 2015), the oxidation pattern of cysteine residues in other lens proteins was reproducible and found to share very similar pattern from human to mouse and between in vitro and in vivo conditions. These results further support the important role of oxidant stress in age-related cataract formation, and in particular the importance of GSH homeostasis as all three systems under investigation were characterized by low (in the lens nucleus) to absent GSH. From a therapeutic perspective, the recapitulation of the protein chemistry changes via in vitro modeling indicates that pharmacological GSH mimetics are needed to protect the aging lens from oxidation. In that regard, the above work represents, to our knowledge, the first comprehensive database on protein disulfides of noncrystallin proteins in aged and cataractous lenses.

Materials and methods

Animals

All animal experiments were conducted in accordance with procedures approved by the Case Western Reserve University Animal Care Committee and conformed to the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. Animals were housed under diurnal lighting condition and allowed free access to food and water. Lens conditional γ-glutamyl cysteine ligase catalytic subunit (Gclc) knockout mice were created as previously described by us and named as LEGSKO mouse (20).

Human and mouse lens samples

All human tissues used for this study were approved by Case Western Reserve University and Sun Yat-sen University Institutional Review Board (IRB). Three types of human lens nuclei were used in this study after

Fig. 4 Representative enzymes whose cysteine residues are involved in disulfide cross-linking. (A) The disulfide bond ratio of the Cys247 in GAPDH in human lens. (B) The disulfide bond ratio of the Cys271 in GAPDH in both LEGSKO mouse and in vitro oxidation by H2O2 compared to their control. In addition, Cys48 in GAPDH was also found close to 40 times oxidized in LEGSKO vs. age-matched WT mice lenses. (C) The disulfide bond ratio of the Cys126 and Cys186 in ALDH1A1 in human lens. (D) The Cys126 and Cys187 (same motif as human Cys186) were found significantly oxidized in LEGSKO vs. WT and also positively associated with in vitro oxidation conditions. In addition, Cys370 oxidation was also detected in LEGSKO mouse lenses. See Fig. 3 legend for labels. All ICAT data are expressed as the ratio vs. young lenses pool, LEGSKO vs. age-matched WT, or oxidized vs. nonoxidized lens protein extract. Standard errors were calculated from three biological replicates. One-way ANOVA was used to compare the significance between groups, and P < 0.05 was considered significant. The significance level was marked by either **, *** or lower case letters and the significance value was shown inside the figures.
careful removal of ~1 mm cortical layer. Three young normal lens nuclei were pooled to serve as control for entire human lens study. The young and aged normal lenses at age 3, 7, 15, 68, 72, and 74 years were collected from the Cleveland Eye Bank/Midwest Eye Bank within postmortem interval of 2–8 h (average, 4.1 h). Human cataract lenses from patients undergoing extracapsular cataract extraction (ECCE) surgery were collected from Huichang County People’s and Ganzhou City People’s Hospital in Jiangxi, China, after obtaining informed consent from the patients. The cataract severity was graded based on Lens Opacities Classification System (LOCS) III. Five grades were given (from I to V) to categorize the severity of cataract with higher grade corresponding to higher severity. Only grade II to V lenses were collected in this study. The ages of the grade II group were 64, 67, and 60 years; the grade III were 60, 59, and 69 years; the grade IV were 56, 63, and 77 years; and the grade V were 75, 80, and 78 years. All cataract lenses were processed within 30 min after surgery to prevent artefactual oxidation by homogenization in 10% trichloroacetic acid (TCA) and 100 mM iodoacetamide (IAM) immediately after receipt. All processed human lens samples collected in China were shipped to Cleveland for ICAT labeling and proteomics study after initial blocking of the free sulfhydryl groups. The details on lens nucleus isolation and pre-ICAT preparations are described in our previous report (Fan et al., 2015). For the mouse lens study, 9-month-old LEGSKO whole lenses with nuclear cataract and age-matched WT whole lenses were used. Three age-matched wild-type whole lenses were pooled to serve as control for the LEGSKO cataract lenses.

**In vitro modeling of lens protein disulfide cross-linking**

The mouse lens protein extract from 3-month-old lenses was incubated with or without 1 or 5 mM hydrogen peroxide (H2O2) in Chelex-treated 50 mM potassium phosphate buffer (pH 7.4) containing 1 mM DTPA at 37 °C for up to 12 h. The samples at 2 and 12 h were taken and processed the same way as human lenses as described above.

**Quantitative Isotope-coded Affinity Tag labeling Experiment (ICAT)**

For ICAT-based quantification by mass spectrometry, 100 μg of protein aliquot of lens sample was reduced by 10 mM tris-(2-carboxyethyl)phosphine (TCEP) before labeling with cleavable ICAT reagent (AB Sciex, Foster City, CA, USA) as described in the

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instruction manual. All human aged normal and aged cataract and LEgSko mouse cataract lens samples were labeled with the cleavable heavy ICAT reagent (13C-ICAT), whereas the young human and age-matched wild-type mouse lens control samples were labeled with the cleavable light ICAT reagent (12C-ICAT). After labeling, a 1:1 mixture of each aged or cataract lens sample with control sample was prepared. The proteins were digested sequentially by lysyl endopeptidase (Wako, Richmond, VA, USA) and trypsin (Promega, Madison, WI, USA), and the peptides were purified sequentially through Oasis HLB Plus column (Waters, Milford, MA, USA) and POROS 50S column (Thermo Fisher, Grand Island, NY, USA). The disulfide bonding peptides were enriched and further purified by avidin affinity column (Thermo Fisher). The biotin group in dried sample was removed by trifluoroic acid (TFA) before LC-MS analysis. A more detailed description of ICAT labeling and purification procedures can be found in our previous report (Fan et al., 2015).

Quantification of proteins by dimethyl labeling

The purpose of the dimethyl labeling was to normalize the ICAT ratios to adjust for differences in the abundance of the cysteine containing proteins between samples. Aliquots (100 µg) from the same original pool of samples that were subjected to the ICAT labeling were digested, and the peptides were purified as described in our previous report (Fan et al., 2015). The tryptic peptides from control sample were labeled with normal formaldehyde and tryptic peptides from aged and cataract samples were labeled with deuterium-labeled formaldehyde (Sigma-Aldrich, St. Louis, MO, USA), respectively, according to previous report (Garcia-Santamarina et al., 2011). After labeling, a 1:1 mixture of each aged or cataract lens sample with control sample was prepared and purified for LC-MS/MS study.

Mass spectrometry (MS) analysis of ICAT- and dimethyl-labeled samples

Peptides were analyzed by liquid chromatography–tandem mass spectrometry (LC-MSMS) using Orbitrap Elite Hybrid Mass Spectrometer (Thermo Electron, San Jose, CA, USA) coupled with a Waters nanoAcquity UPLC system (Waters, Taunton, MA, USA). The peptides were separated using a linear gradient of acetonitrile in 0.1% formic acid from 2% to 35% for 60 min and then up to 90% for 15 min prior to column equilibration with water in 0.1% formic acid for 15 min, making a total run-time of 90 min. The spectra were acquired in the positive ionization mode by data-dependent methods consisting of a full MS scan in high-mass accuracy FT-MS mode at 120 000 resolution, and MS/MS on the twenty most abundant precursor ions in CID mode with the normalized collision energy of 35%. Mascot Daemon (version 2.4.0; Matrix Science) was used to identify the peptides, and the data were searched against SwissProt human or mouse database. The mass tolerance was set at 10 ppm for precursor ions and 0.8 Da for product ions. For dimethyl labeling, the light (with regular formaldehyde) and intermediate (with deuterated formaldehyde)-labeled peptides at their N-terminal and lys residues (with the mass shift of 28.0313 and 32.0475 Da, respectively) in addition to carbamidomethylation of Cys and oxidation of Met residues were set at as variable modifications for Mascot Daemon. Excluding crystallin peptides, peptides with light labeling and intermediate or heavy labeling in each protein were analyzed and the ratio of light to heavy dimethylation modification was calculated by manual extraction of each peptide on the basis of peptide peak heights. For ICAT labeling, the light and heavy ICAT-labeled peptides with mass shift of 227.1270 and 236.1571 Da, respectively, were first identified through Mascot Demon and then confirmed via manual examinations. The average ratio from multiple spectra covering the same sites of particular peptide was calculated in each sample, and the average ratio from three samples after adjustment from dimethyl labeling results was reported as final ICAT quantification results. For both ICAT- and dimethyl-labeled samples, Mascot ions score threshold is set at 20. The false discovery rate of peptide matching is identified within 3%. The detailed procedures of ICAT-labeled peptide identification and quantification have been reported in our previous study (Fan et al., 2015). All reported ICAT ratios were identified with the standard error < 0.1 and correlation coefficiency > 0.99 as described in previous report (Leung et al., 2005). The mass spectrometry proteomics data have been deposited to the ProteomeXchange consortium via the PRIDE partner repository with the dataset identifier PXD002658 and 10.6019/PXD002658.

Statistical methods

All values were expressed as means ± SE. Statistical analysis was performed according to the methods previously described in detail in Sell et al. (2000). In brief, statistical significance of differences in mean values was assessed by repeated-measures ANOVA or Student’s t-test. P-values of < 0.05 were considered statistically significant.

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Author contributions

XF and VMM conceived the project. XF, BW, GH, and SZ conducted the experiments, and MG, BL, and JY supplied the essential materials. XF, BW, and VMM interpreted the data. XF and VMM wrote the manuscript.

Conflict of interest

None declared.

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