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Protein Cysteines Map to Functional Networks According to Steady-state Level of Oxidation

Young-Mi Go[1], Duc M. Duong[2], Junmin Peng[2] and Dean P. Jones[1]*

[1]*Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, Emory Proteomics Service Center, Emory University, Atlanta, Georgia 30322 USA*
[2]*Department of Human Genetics, Center for Neurodegenerative Diseases, Emory Proteomics Service Center, Emory University, Atlanta, Georgia 30322 USA*

Abstract

The cysteine (Cys) proteome serves critical roles in protein structure, function and regulation, and includes key targets in oxidative mechanisms of disease. Thioredoxins maintain Cys residues in thiol forms, and previous research shows that the redox potential of thioredoxin in mitochondria and nuclei is more reduced than cytoplasm, suggesting that proteins in these compartments may have different steady-state oxidation. This study measured fractional oxidation of 641 peptidyl Cys residues from 333 proteins in HT29 cells by mass spectrometry. Average oxidation of cytoplasmic, nuclear and mitochondrial proteins was similar (15.8, 15.5, 14%, respectively). Pathway analysis showed that more reduced cytoplasmic Cys were in proteins associated with the cytoskeleton, more reduced nuclear Cys with Ran signaling and RNA post-transcriptional modification, and more reduced mitochondrial Cys with energy metabolism, cell growth and cell proliferation. More oxidized cytoplasmic Cys included associations with PI3/Akt, Myc-mediated apoptosis and 14-3-3-mediated signaling. Weaker associations of oxidized nuclear and mitochondrial Cys occurred with granzyme B signaling and intermediary metabolism, respectively. Thus, steady-state peptidyl Cys oxidation is associated with functional pathways rather than simply with organellar distribution. This suggests that oxidative mechanisms of disease could target functional pathways or networks rather than individual proteins or subcellular compartments.

Keywords: Redox proteomics; Cysteine oxidation; Functional network; Ingenuity pathway analysis; Compartmentalized redox signaling

Abbreviations: Cys: Cysteine; CySS: Cystine; GSH: Glutathione; ICAT: Isotope-Coded Affinity Tag; IPA: Ingenuity Pathway Analysis; LC/MS: Liquid Chromatography-Mass Spectrometry; Trx: Thioredoxin

Introduction

Cysteine (Cys) residues are present in most mammalian proteins and are critical for many cell functions [1]. Oxidation and other chemical modifications of Cys residues are commonly considered in disease mechanisms [2]. A small number of studies provide evidence that modification of specific thiols can be mechanistically important. For instance, microinjection into endothelial cells of thioredoxin-1, modified at Cys73 by reaction with a stoichiometric amount of acrolein, resulted in stimulation of monocyte adhesion, an early step in atherogenesis [3]. Evidence for thiol switches in cell signaling, transcriptional activation, cell survival and apoptotic signaling [4] suggest that additional specific targets may exist. On the other hand, studies of protein glutathionylation [5], nitrosylation [6], sulfenylation [7] and alkylation [8,9] show that hundreds of protein thiols are reactive and suggest that targeting of single thiols in disease mechanisms may be rare.

A common alternative interpretation is that a subset of thiols is selectively sensitive due to compartmental localization. For example, loss of mitochondrial GSH parallels cell death in hepatocytes [10], is potentiated by selective depletion of mitochondrial GSH [11] and is a characteristic of alcoholic liver disease [12]. More recent studies of nuclei show that depletion of nuclear GSH blocks cell proliferation [13] and that nuclear proteins are more resistant to oxidation [14]. Measurement of steady-state oxidation of Trx in subcellular compartments [15] showed an increasing order of oxidation, cytoplasm > nuclei > mitochondria [1], suggesting that a similar pattern of sensitivity to oxidative stress could occur. Importantly, these accumulating data show that many proteins exist with Cys residues partially oxidized, i.e., under steady-state conditions where the rates of reduction are insufficient to maintain the fully reduced form at *in vivo* oxidation rates [15]. This non-equilibrium state has been noted in proteomics analyses by Le Moan et al. [16] and also found in targeted analyses of thioredoxin-1 (Trx-1), mitochondrial Trx-2, thioredoxin reductase-1, thioredoxin reductase-2, peroxiredoxin-1, peroxiredoxin-3, NF-κB (p50), redox factor-1, protein disulfide isomerase and other proteins [for summary, see [1,17]]. Such data are inconsistent with older ideas that thiol oxidation largely represents an artifact of cell extraction [18].

Processing of proteins within the secretory pathway is widely recognized as a kinetically limited process in which disruption of normal oxidative processes can activate a cell death response [19]. Measurements of steady-state oxidation of proteins show that during processing, protein disulfide isomerase is partially oxidized. Similarly, several key transcription factors have a critical Cys residue in the DNA binding region that provides a redox switch to inhibit activity when the Cys is oxidized [20-23]. Thiol antioxidants such as Trx-1 and GSH function in opposition to this oxidation to maintain activity [23,24]. Dynamic redox control by this mechanism is indicated by functional data for NF-κB in living cells. For instance, NF-κB reporter activity is decreased by generation of H_2O_2 within nuclei [24] and increased by targeting peroxiredoxin-1, a Trx-1-dependent peroxidase, to nuclei

***Corresponding author:** Dean P. Jones, Emory University, 205 Whitehead Research Center, Atlanta, GA 30322. Tel: 404-727-5970; Fax: 404-712-2974; E-mail: dpjones@emory.edu

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[25]. Additional data show functional redox responses of Trx-1 and Trx-2 in signaling by EGF, TNF-α and extracellular redox potential, and also in response to metabolic precursors and Nrf-2 activators [reviewed in [1,15,17]]. Thus, substantial evidence exists that specific thiol redox systems function in cells in a dynamically responsive, partially oxidized state.

As a basis to obtain a more global understanding of physiologic and pathophysiologic protein oxidation, we developed a proteomic method to measure the fractional oxidation of specific Cys residues in proteins [26,27]. This method is similar to the pioneering research of Sethuraman et al. [28], Le Moan et al. [16] and Leichert et al. [29] to examine protein oxidation during oxidative stress. In our approach, proteins are rapidly extracted and treated with a heavy stable isotopic form of the Isotope-Coded Affinity Tag (ICAT) reagent [30], followed by removal of the reagent, treatment with reductant and treatment with the light form of the reagent. With liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS), this allows measurement of the percent oxidation of specific residues in peptide sequences. The approach is relatively reliable for peptide sequence identification because it requires that the sequence be identified twice (as the H-form and the L-form) for inclusion. In the present study, we have further required that the data be replicated in multiple biologic experiments for inclusion.

Many proteins contain very similar primary structure so that the protein source of a peptide cannot be ascertained by the sequence information alone. None-the-less, proteins of origin can be inferred based upon matches in database searches. Such matches can be useful in network analysis even if some are incorrect because pathway analysis tools include statistical analyses of functional network associations as indices of the likelihood of random association. Detailed discussions of the methods and limitations are available (http://www.ingenuity.com). Subsequent use of the inferred redox pathways and networks retain the limitation that there is no independent proof that the measured peptides are derived from proteins with matching amino acid sequences.

In research focused on capture of steady-state redox values for kinetically limited thiol systems, the caveat must be emphasized that all measurements of biological thiols involving extraction and chemical modification are operational in the sense that they can only reflect the efficiency of alkylation relative to oxidation in trapping the thiol form [See Hansen and Winther [31] for detail discussion of methods]. Gilbert [32] discussed potential artifacts in a detailed review on the evidence that the steady-state GSH/GSSG redox potential in rat liver is relatively oxidized compared to its physiologic reductant, NADPH/$NADP^+$. His conclusion reinforced that of Sies and Summer [33] that the endogenous rates of oxidation maintain a non-equilibrium steady state. We have also studied this point extensively for GSH and other thiol/disulfide couples in different cell types, subcellular compartments and in vivo [see [1,15,17,34] and references therein] and confirm this conclusion. A number of methods papers can be consulted for additional consideration of pitfalls in measurement of biological thiols [e.g. [31]], but the most salient points are 1) extractions need to be performed rapidly under conditions to minimize extent of both oxidative and reductive artifacts, 2) artifacts can be minimized by extraction at 0°, a condition where oxidation rates are slower than occurring at room temperature or in vivo (37°), 3) prolonged storage, even at -80°, should be avoided because it can result in protein oxidation and 4) many experiments in the literature have oxidants or catalytic metal ions added as part of the experimental design; such experiments are prone to oxidation during extraction and analysis, especially for prolonged storage or analysis times. In contrast, in the absence of added oxidants and metals, endogenous oxidants are present at low concentrations, diluted during extraction and do not support thiol oxidation at rates comparable to rates of alkylation by thiol-reactive reagents present at many orders of magnitude higher concentration.

The present research used LC-MS/MS-based redox proteomics to test the hypothesis that steady-state oxidation of proteins differs among cellular compartments and test for possible associations with functional pathways. HT29 cells were used because redox systems in these cells, including the mitochondrial and nuclear thioredoxin and GSH systems, are well characterized and known to differ in steady-state redox potentials [14,34-36]. Unexpectedly, the results did not show a pattern of mitochondria and nuclei Cys residues being more reduced than in cytoplasmic proteins. Rather, the results show that Cys residues with higher and lower percent oxidation exist in different functional pathways within compartments. Thus, the data indicate that oxidative mechanisms of disease and toxicity may involve disruption of functional redox networks rather than individual proteins or subcellular compartments.

Materials and Methods

Cell culture and protein extraction

HT-29 cells purchased from ATCC were cultured in 10% FBS in DMEM (37°C, 5% CO_2). Mouse aortic endothelial cells (MAEC) obtained from the thoracic aortas of C57/BL6 mice were isolated [26], cultured in DMEM containing 20% FBS, 100 μg/mL endothelial cell growth supplement (Biomedical Technologies Inc.), and 2.5 U/mL heparin. Cells with 90% confluency were washed with ice-cold PBS, protein was precipitated with ice-cold 10% TCA and samples were processed as described below for redox ICAT analysis using ICAT reagents (Invitrogen/Applied Biosystems). Previous studies of thiol-disulfide exchange and efficiency of thiol trapping with timed sequences of extraction and alkylation with different thiol reagents show that this extraction approach is suitable to estimate steady-state levels of thiol oxidation [34,37-39].

Redox proteomic analysis

Redox ICAT was performed using Isotope Coded Affinity Tag (ICAT)-based mass spectrometry [26,27]. Briefly, protein precipitate (120 μg) was washed with ice-cold acetone, resuspended in denaturing buffer provided by the manufacturer and treated with the biotin-labeled thiol reagent [Heavy isotopic (H-ICAT)] for 1 h at room temperature. Protein was then precipitated by 10% TCA for 30 min on ice, pelleted by centrifugation, washed with acetone, and resuspended in denaturing buffer. Unlabeled oxidized forms (e.g., disulfides) in the proteins were then reduced by TCEP [tris-(2-carboxyethyl phosphine)] and finally labeled with the second biotin-labeled thiol reagent (Light ICAT, L) for 1 h. Samples were digested with trypsin for 18 h, fractionated by cationic exchange followed by avidin purification, and analyzed by mass spectrometry as described below. Peptides were identified with an H to L ratio as a measure of the reduced/oxidized state of protein, expressed as percentage values, and labeled as "% reduced state or % oxidized state". Identified peptides were individually processed to eliminate redundancies, matched to proteins based upon amino acid sequences and assigned to subcellular compartments based upon Ingenuity Pathway Analysis (IPA), Uniprot database annotation and scientific literature searches of the corresponding proteins.

Mass spectrometry

Peptides were analyzed by reverse-phase liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) [40]. Peptide eluates were monitored in a MS survey scan followed by ten data-dependent MS/MS scans on an LTQ-Orbitrap ion trap mass spectrometer (Thermo Finnigan, San Jose, CA). The LTQ was used to acquire MS/MS spectra (2 m/z isolation width, 35% collision energy, 5,000 AGC target, 150 ms maximum ion time). The Orbitrap was used to collect MS scans (300-1600 m/z, 1,000,000 AGC target, 750 ms maximum ion time, resolution 60,000). All data were converted from raw files to the .dta format using ExtractMS version 2.0 (Thermo Finnigan, San Jose, CA). The acquired MS/MS spectra were searched against a concatenated target-decoy human reference database of the National Center for Biotechnology Information using the SEQUEST Sorcerer algorithm (version 3.11, SAGE-N) [41]; Searching parameter including: partially tryptic restriction, parent ion mass tolerance (± 50 ppm), product ion tolerance (± 0.5 m/z), dynamic modifications of oxidized Met (+15.9949 Da), and differential ICAT modified Cys (+9.0302 Da), and static ICAT modification of Cys (+227.1270 Da). The peptides were classified by charge state and tryptic state (fully and partial) and first filtered by mass accuracy (10 ppm for high-resolution MS), and then dynamically by increasing XCorr and ΔCn values to reduce protein false discovery rate to less than 1%, according to the target-decoy strategy [42,43]. Parameter settings allowed detection of 100% oxidation and 100% reduction as well as H:L ratios for partially oxidized peptidyl Cys. Peptides included for analyses are presented in Supplement 1.

Quantification of protein thiols

HT-29 cells grown in 6-well plate and mouse liver were extracted as described above for the ICAT procedure and analyzed for thiol content using DTNB [44] and expressed relative to protein content (Biorad DC Assay).

Biological network and pathway analysis by IPA

Functional genomic and proteomic networks affected by protein redox state were analyzed using IPA (www.ingenuity.com) [45,46]. Protein peptide redox data were converted from H:L ratio to fold reduction (or oxidation) compared to average reduction (oxidation) for input. IPA scores for each network are derived from a p-value and indicate the likelihood of the focus peptides (proteins) in a network being found together due to random chance. Scores of 2 or higher have at least a 99% confidence of not being generated by random chance alone; higher scores indicate a greater confidence. We presented top scored networks in the figures; IPA network scores are provided in the text. Information on other significant networks is provided in Table 2. More detailed description of IPA-identified pathway names can be found in http://people.mbi.ohio-state.edu/baguda/PathwayAnalysis/ipa_help_manual_5.5_v1.pdf.

Results

General characteristics

Average oxidation of Cys residues from all identified peptides was similar for 5 independent experiments [21.6% (n = 600), 14.4% (n = 958), 21.1% (n = 719), 15.0% (n = 796), 13.7% (n = 374)]. Data compiled for peptides present in at least 3 of the experiments included 641 peptides associated with 333 proteins. Average oxidation was 15.7% (Figure 1A), with a median oxidation of 12.3%. Average data for each peptide are provided along with associated protein name and descriptive statistics (SD and SEM) in Online Supplement 1, sorted according to subcellular compartment (A, cytoplasm; B, nuclei; C, mitochondria).

Protein synthesis utilizes only reduced Cys, so even though many proteins are oxidized during protein processing and post-translational modification [47], we expected that most peptides would be >99% reduced. The results showed that only 6.9% of peptides were >95% reduced (Figure 1B). To determine whether the ICAT labeling procedure introduced excessive oxidation, we used a direct chemical approach to measure percent oxidation of total cellular protein thiols. Cells were extracted under conditions used for ICAT labeling, and the protein thiol content was measured using DTNB (5,5'-dithio-bis(2-nitrobenzoic acid) before and after reduction with TCEP (tris-(2-carboxyethyl)phosphine). Results showed 86.0% reduction (Figure 2A), which was comparable to the average value for all peptides (84.3%) measured by mass spectrometry. To compare to *in vivo* reduction of proteins, mouse liver proteins were similarly analyzed and showed 91.7% reduction (Figure 2B), indicating that average reduction of proteins *in vivo* is greater than in the cell culture. Such a difference could occur due to higher O_2 concentration and/or to lower GSH concentrations in cell culture. None-the-less, the ICAT-labeling procedure captured the overall character of cellular protein Cys oxidation, but *in vitro* cell conditions may have greater percent oxidation than found *in vivo*.

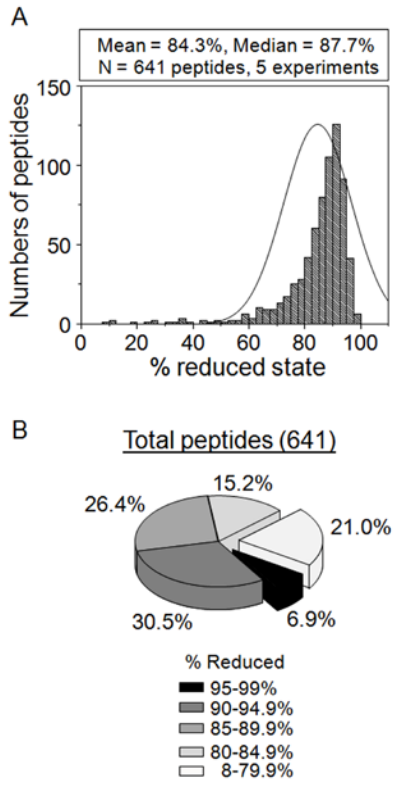

Figure 1: Distribution of reduced states of peptidyl Cys residues in HT29 cells. A. Histogram showing number of peptides detected according to % reduction of Cys measured by sequential labeling according to the redox ICAT method. B. Pie chart showing the distribution of peptides according to the measured percent reduction. Note that only 6.9% were >95% reduced while 21.0% were >20% oxidized.

Oxidation of proteins in cytoplasm, nuclei and mitochondria

Assignments of peptides to proteins and proteins to compartments were based upon database and literature searches. While there are possible errors due to splicing variants and the presence of proteins in more than one compartment, the data capture enough peptides (cytoplasm, 498; nuclei, 106; mitochondria, 37) and proteins (cytoplasm, 265; nuclei, 49; mitochondria, 19) to estimate compartmental oxidation. In contrast to previous data obtained for the thioredoxins, the average percent oxidation of peptides was the same for all compartments measured (cytoplasm, 15.8 ± 3.0; nuclei, 15.5 ± 3.0; mitochondria, 14.0 ± 2.6). Previously published data shown that Trx2, when expressed in the cytoplasm as a truncated form without mitochondrial targeting sequence, existed with a greater percent oxidation than the same protein in the mitochondria when analyzed with comparable extraction methods [48]. Similarly a nuclearly targeted Trx1 showed less steady-state oxidation than Trx1 in cytoplasm [26]. Thus, the data show that the average protein oxidation in HT29 cells differs from that expected from the data for thioredoxins.

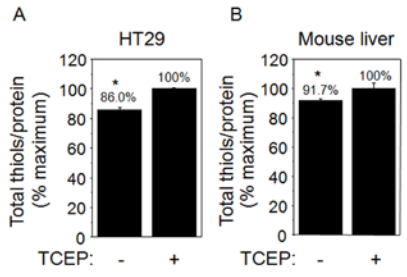

Figure 2: Percent reduction of total Cys *in vitro* in HT-29 cells and *in vivo* in mouse liver. A. HT-29 cells were extracted and protein thiol content was measured with DTNB with and without treatment by TCEP. Results showed similar percent reduction to the average found for the ICAT method (see Figure 1). B. Mouse liver was extracted and treated similarly to determine average protein thiol oxidation *in vivo*. Values in A are mean SEM: untreated, 86.0 1.5; TCEP-treated, 100 0.9, n = 3-5. Values in B are untreated, 91.7 ± 1.5 and TCEP-treated, 100 ± 4.0, n = 3. *p < 0.05.

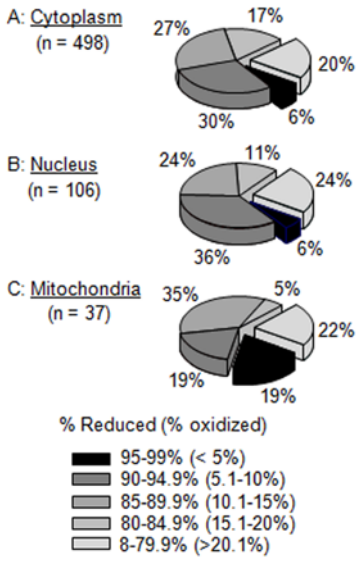

Figure 3: Percentages of peptidyl Cys reduction according to subcellular distribution. Peptides were assigned to either cytoplasmic, nuclear or mitochondrial compartments based upon IPA, Uniprot and literature searches. While the proportion of relatively oxidized Cys (about 20%) is similar among the compartments, a much greater percent of the mitochondrial Cys (19%) are highly reduced compared to the other compartments (about 6%). Note that the compartmental assignments represent only an approximation because some proteins have multiple distributions and others are more specifically associated with other subcellular structures. A. Cytoplasm. B. Nucleus. C. Mitochondria. See Online Supplement 1 for detailed data.

Examination of the variation of percent reduction among the peptides in the compartments showed that mitochondria had the greatest percentage of highly reduced peptides with nearly 20% being >95% reduced (Figure 3C), contrasted by data for peptides of cytoplasmic and nuclear proteins, where only 6% were >95% reduced (Figure 3A, 3B). A similar fraction of proteins was present at <80% reduced. This pattern is consistent with previous studies of Trx-2 redox state in mitochondria, which is more reduced than cytoplasmic and nuclear Trx-1 under control conditions but also more sensitive to oxidation in response to imposed oxidative challenge or TNF-α signaling. If one stratifies differently, e.g., combining those >90% reduced, there is no apparent difference in percentage oxidation between the compartments. In either case, however, the data suggest that control of peptide oxidation within a compartment is not governed by a global, uniform mechanism but rather that subsets of peptides within a compartment are differentially regulated.

Evolutionary conservation among vertebrates of oxidized and reduced peptidyl Cys

A critical but difficult question is whether the 641 peptidyl Cys detected are functional or non-functional. Pathway analysis assumes that functionally important associations are present. With over 200,000 total Cys encoded by about 20,000 genes, one can estimate that there are about 10 Cys per protein. Catalytic sites of proteins can account for only a small fraction, so most of the Cys must have no specific function other than space filling, or be involved in other functions, such as structure, regulation or non-specific protection against oxidative stress [49]. Mass spectrometry-based proteomics is biased toward high-abundance proteins so that if most of the Cys have no specific functions, global differences in random Cys in high-abundance proteins could provide an artifactual impression of functional networks. To examine this, we assumed that non-functional Cys would have a greater tendency to be random and not conserved with evolution while functional Cys would be conserved. We examined evolutionary conservation of the 10 most oxidized (>39.3% oxidized, CD59, CD59 glycoprotein; EIF2S3, eukaryotic translation initiation factor 2 subunit 3; EPCAM, epithelial cell adhesion molecule; LAP3, cytosol aminopeptidase; FASN, fatty acid synthase; HNRNPR, heterogeneous nuclear ribonucleoprotein R; HSP90AA1, heat shock protein HSP 90-alpha; HSP90AB1, HSP90AB1 protein; NOP56, nucleolar protein 56; RAC1, Ras-related C3 botulinum toxin substrate 1; see Supplement 1) and 10 most reduced peptidyl Cys (>97.0% reduced, ACTB, beta-actin; DDX39, DEAD box polypeptide 39; EEF2, elongation factor 2; HNRNPD, heterogeneous nuclear ribonucleoprotein D; MDH2, malate dehydrogenase-2; PKM2, pyruvate kinase isozymes M1/M2; RAC1, Ras-related C3 botulinum toxin substrate 1; RPL3, 60S ribosomal protein L3; SDHA, succinate dehydrogenase subunit A; XRCC5, X-ray repair cross-complementing protein 5; see Supplement 1) by searching the Uniprot database for corresponding proteins in 5 mammalian species [human, Sumatran orangutan (*Pongo abelii*), bovine, mouse, rat] and 5 other vertebrate

Protein	CYS	Hum	Oran	Bovin	Rat	Mous	Chick	X. trop	X laev	D rerio	S salar	Total	Cons	% Cys cons
Ten most reduced peptides														
SDHA	536	+	+	+	+	+	+	+	+	+	+	10	10	100
ACTB	285	+	+	+	+	+	+	+	+	+	+	10	10	100
RPL3	114	+	0	+	+	+	+	+	+	+	+	9	9	100
PKM2	473	+	+	+	+	+	0	-	+	-	-	9	6	67
MDH2	89	+	+	+	+	+	+	-	-	-	-	10	6	60
EEF2	290	+	+	+	+	+	+	+	0	-	-	9	7	78
RAC1	105	+	0	+	+	+	+	+	+	+	+	9	9	100
DDX39	197	+	0	+	+	+	+	+	+	+	+	9	9	100
HNRNPD	126	+	0	+	+	+	+	+	+	+	+	9	9	100
XRCC5	489	+	+	+	+	+	0	0	+	+	0	7	7	100
Ten most oxidized peptides														
HSP90AB1	410	+	+	+	+	+	+	+	+	+	+	10	10	100
CD59	70	+	+	+	+	+	+	0	0	0	+	7	7	100
LAP3	462	+	0	+	+	+	+	+	+	+	+	9	9	100
HNRNPR	226	+	+	+	+	+	+	-	-	+	+	10	8	80
HSP90AA1	420	+	+	+	+	+	+	+	+	+	+	10	10	100
FASN	212	+	0	+	+	+	+	0	0	0	0	5	5	100
NOP56	384	+	+	+	+	+	+	+	+	+	-	10	9	90
RAC1	6	+	+	+	+	+	+	+	+	+	+	10	10	100
EPCAM	99	+	0	+	+	+	+	+	+	+	+	9	9	100
EIF2S3	236	+	+	+	+	+	+	+	+	+	+	10	10	100
Ten randomly selected from measured peptides														
DDT	24	+	**0**	+	+	+	+	+	+	+	+	9	9	100
PKM2	358	+	+	+	+	+	+	+	+	+	+	10	10	100
GBLP	207	+	**0**	+	+	+	+	+	+	+	+	9	9	100
AL1A1	186	+	+	+	+	+	+	+	+	+	+	10	10	100
ALDOC	178	+	+	+	+	+	**0**	+	+	+	+	9	9	100
AAAT	467	+	**0**	+	+	+	**0**	+	+	+	**0**	7	7	100
NASP	708	+	**0**	+	+	+	+	+	+	-	-	9	7	78
IMB1	158	+	**0**	+	+	+	**0**	**0**	+	**0**	**0**	5	5	100
GCN1L	648	+	**0**	**0**	**0**	+	**0**	**0**	**0**	-	**0**	3	2	67
PARK7	53	+	**0**	+	+	+	+	+	+	+	+	9	9	100
Avg % for proteins														94
Avg % for species		100	100	100	100	100	100	86	92	80	80	261	246	94

*Abbreviations: AAAT, neutral amino acid transporter B(0); ACTB, beta-actin; AL1A1, retinal dehydrogenase 1 (aldehyde dehydrogenase family 1 member A1); ALDOC, fructose-bisphosphate aldolase C; CD59, CD59 glycoprotein; DDX39, DEAD box polypeptide 39; DDT, D-dopachrome decarboxylase (D-dopachrome tautomerase), EEF2, elongation factor 2; EIF2S3, eukaryotic translation initiation factor 2 subunit 3; EPCAM, epithelial cell adhesion molecule; LAP3, cytosol aminopeptidase; FASN, fatty acid synthase; GBLP, guanine nucleotide-binding protein subunit beta-2-like 1; GCN1L, translational activator GCN1; HNRNPD, heterogeneous nuclear ribonucleoprotein D; HNRNPR, heterogeneous nuclear ribonucleoprotein R; HSP90AA1, heat shock protein HSP 90-alpha; HSP90AB1, HSP90AB1 protein; IMB1, importin subunit beta-1 (karyopherin subunit beta-1); MDH2, malate dehydrogenase-2; NASP, nuclear autoantigenic sperm protein; NOP56, nucleolar protein 56; PARK7, protein DJ-1 (Parkinson disease protein 7); PKM2, pyruvate kinase isozymes M1/M2; RAC1, Ras-related C3 botulinum toxin substrate 1; RPL3, 60S ribosomal protein L3; SDHA, succinate dehydrogenase subunit A; XRCC5, X-ray repair cross-complementing protein 5

Table 1: Conservation among vertebrates of most reduced and most oxidized peptidyl Cys*.

Cytoplasm	
Reduced	
1. Cancer, Gastrointestinal Disease, Genetic Disorder	58
2. Cellular Assembly and Organization, Protein Synthesis, Cell Death	
3. Protein Synthesis, Cancer, Gastrointestinal Disease	30
4. Connective Tissue Disorders, Inflammatory Disease, Skeletal and Muscular Disorders	25
5. Cell Morphology, Cardiovascular System Development and Function, Gene Expression	23
Oxidized	
1. Cancer, Cellular Function and Maintenance, Embryonic Development	51
2. Cell Signaling, Post-Translational Modification, Protein Folding	39
3. Post-Translational Modification, Protein Folding, Cellular Compromise	37
4. Cellular Compromise, Cellular Development, Embryonic Development	31
5. Gene Expression, Cellular Assembly and Organization, Connective Tissue Disorders	22
Nuclei	
Reduced	
1. RNA Post-Transcriptional Modification, Gene Expression, Infection Mechanism	32
2. Molecular Transport, RNA Trafficking, Cellular Compromise	26
Oxidized	
1. Cellular Assembly and Organization, Cancer, Cell Morphology	35
2. RNA Post-Transcriptional Modification, Cellular Assembly and Organization, Cellular Movement	35
Mitochondria	
Reduced	
1. Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry	24
2. Genetic Disorder, Metabolic Disease, Cancer	14
Oxidized	
Cellular Function and Maintenance, Cell Cycle, Reproductive System Development and Function	36
IPA-calculated significant score for each network is generated using a p-value calculation, and is displayed as the negative log of that p-value. This score indicates the likelihood that the assembly of a set of focus genes in a network could be explained by random chance alone. (Note provided by IPA: A score of 2 indicates that there is a 1 in 100 chance that the focus genes are together in a network due to random chance. Therefore, networks with scores of 2 or higher have at least a 99% confidence of not being generated by random chance alone. http://www.ingenuity.com/products/Monarch.NovelMolecularMechanisms_high.pdf)	

species [chicken (*Gallus gallus*), *Xenopus laevis, Xenopus tropicalis*, zebrafish (*Danio rerio*) and Atlantic salmon (*Salmo salar*)]. The results showed that all 20 Cys residues were completely conserved among the 6 warm-blooded vertebrate species (Table 1). Overall, 93% of the Cys residues (169 out of 181) in these proteins were conserved among the 10 vertebrate species (Table 1). In contrast, there was only 78% identity in amino acid residues between humans and salmon. A similar analysis of 10 randomly selected peptides showed that the Cys residues measured were 96% conserved (Table 1). Although these 30 peptidyl Cys represent only a small subset of the total peptidyl Cys residues measured, conservation of these residues suggests that Cys residues detected by this redox ICAT method are likely to be functional. Accordingly, pathway analysis was used to determine whether oxidized and reduced Cys were associated with functional networks. All top networks identified by Ingenuity Pathway Analysis (IPA) software (www.ingenuity.com) upon reduced and oxidized proteins are listed in Table 2 and network data analyzed by compartment-specific proteins are described in the following sections.

Pathway analysis of cytoplasmic, nuclear, and mitochondrial protein peptides according to percent oxidation

Data were analyzed using IPA to test for associations of relatively oxidized and reduced peptidyl Cys with functional networks. Separate analyses were performed for total cellular peptides and cytoplasmic, nuclear and mitochondrial peptides. The results were qualitatively the same, and only the compartmental data are presented. In these analyses, association of a peptide with a protein was based upon the amino acid sequence and output from the SEQUEST analysis, without the requirement for detection of multiple peptides from the protein. This was considered justifiable because the peptide sequence was measured redundantly and the primary focus was on oxidation of Cys residues in peptides. Although there is a chance of misidentification, the functional networks include many proteins so that networks with a high confidence score are relatively insensitive to misidentification of individual proteins.

Pathway analysis of cytoplasmic peptides. The cytoplasmic dataset included 498 peptides, providing enough data to restrict analysis to the most reduced 20% and most oxidized 20%. The most reduced peptides associated with multiple network functions, including cancer, gastrointestinal disease, cellular assembly and organization and protein synthesis (Figure 4A; Score 58). Because the HT29 cells are a colon cancer cell line, network association with cancer and gastrointestinal disease is expected. However, many cytoskeletal proteins associated in the top network (Figure 4A), indicating that highly reduced Cys are functionally associated with the organizational structure of the cytoskeleton. This suggests a redox organizational structure that

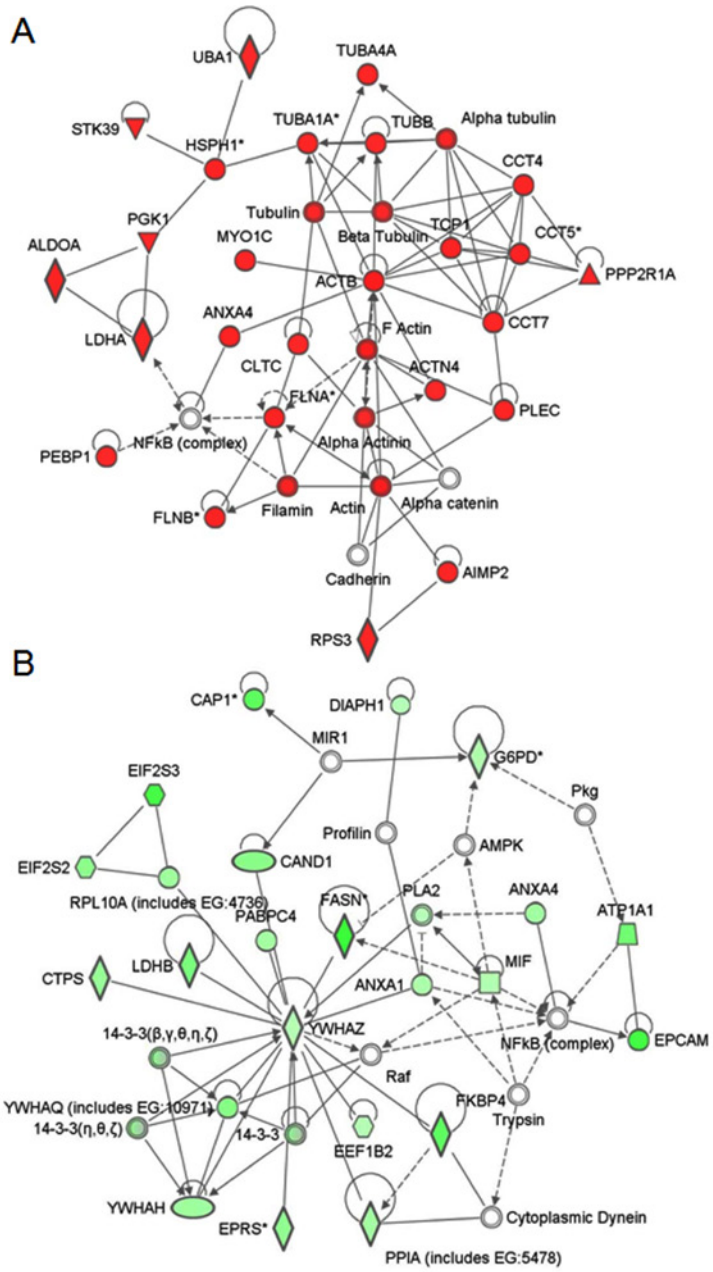

Figure 4: Association of cytoplasmic peptidyl Cys with functional networks according to reduction and oxidation of Cys residues. Percent reduction values for the 20% most reduced Cys (Panel A) and the 20% most oxidized Cys (Panel B) were analyzed by Ingenuity Pathway Analysis (IPA). In this analysis, symbols with colors represent proteins associated with peptides identified with percent oxidation data in Supplement 1. Open symbols represent components of the functional network for which data were not available. Asterisks indicate that multiple identifiers in the dataset file map to a single gene/chemical in the global molecular network.

In A, the top network associations of the most highly reduced Cys were with cancer, gastrointestinal disease, cellular assembly and organization and protein synthesis (Score 58). A large number of these proteins are associated with the cytoskeleton. In B, the most oxidized peptides had network associations with cancer, cell signaling and post-translational modification (Score 51). Abbreviations: ACTB, beta-actin; ACTN4, alpha-actinin-4; AIMP2, aminoacyl tRNA synthase complex-interacting multifunctional protein 2; ALDOA, fructose-bisphosphate aldolase A; AMPK, 5'-AMP-activated protein kinase; ANXA1, annexin A1; ANXA4, annexin A4; ATP1A1, sodium/potassium-transporting ATPase subunit alpha-1; CAND1, cullin-associated NEDD8-dissociated protein 1; CAP1, adenylyl cyclase-associated protein 1; CCT4, T-complex protein 1 subunit delta; CCT5, T-complex protein 1 subunit epsilon; CCT7, T-complex protein 1 subunit eta (TCP-1-eta); CLTC, clathrin heavy chain 1; CTPS, CTP synthase 1; DIAPH1, protein diaphanous homolog 1; EEF1B2, eukaryotic elongation factor 1-beta; EIF2S2, eukaryotic translation initiation factor 2 subunit 2; EIF2S3, eukaryotic translation initiation factor 2 subunit 3; EPCAM, epithelial cell adhesion molecule; EPRS, bifunctional aminoacyl-tRNA synthetase; FASN, fatty acid synthase; FKBP4, peptidyl-prolyl cis-trans isomerase; FLNA, filamin A; FLNB, filamin B; G6PD, glucose 6-phosphate 1-dehydrogenase; HSPH1, heat shock protein 105 kDa; LDHA, L-lactate dehydrogenase A chain; LDHB, L-lactate dehydrogenase B chain; MIF, macrophage migration inhibitory factor; MIR1, E3 ubiquitin-protein ligase; MYO1C, myosin-Ic (myosin I beta); PABPC4, polyadenylate-binding protein 4; PEBP1, phosphatidylethanolamine-binding protein 1; PGK1, phosphoglycerate kinase 1; PLA2, phospholipase A2; PLEC, plectin; PP1A, serine/threonine-protein phosphatase PP1-alpha catalytic subunit; RPS3, 40S ribosomal protein S3; STK39, STE20/SPS1-related proline-alanine-rich protein kinase; TCP1, T-complex protein 1 subunit alpha; TUBA1A, tubulin alpha-1A chain; TUBA4A, tubulin alpha-4A chain; TUBB, tubulin beta; UBA1, ubiquitin-like modifier-activating enzyme 1; YWHAH, 14-3-3 protein eta; YWHAZ, 4-3-3 protein zeta/delta.

could render the cytoskeleton sensitive to oxidation and susceptible to reactive electrophiles, characteristics previously identified by toxicologic research [50,51]. However, in other contexts, actin is more oxidized than other proteins and undergoes reduction under growth factor stimulation and ROS production [52]. Thus redox signaling and control of networks could be dependent upon cell type and functional state, i.e., more selective and complex in mechanism than the concept of a steady-state redox state for a signaling pathway.

To determine whether cytoskeleton-associated proteins were more reduced than average in another cell type and another species, we examined previously published redox proteomic data for mouse aortic endothelial cells (MAEC) exposed to different extracellular cysteine/cystine redox potentials (E_hCySS). E_hCySS of 0 mV and -150 mV were used as most oxidized and most reduced extracellular redox conditions, respectively [26]. Of the cytoskeletal network proteins in Figure 4A, 15 were measured in MAEC. After 1 h culture in media at -150 mV of E_hCySS, the 15 cytoskeletal proteins were 8% more reduced than the average of all MAEC peptides measured (Supplement 2). These data support the conclusion from the human HT29 cell data by showing that in another cell type and another species, peptidyl Cys from cytoskeletal proteins are more reduced than those of other cellular proteins.

The study of effects of extracellular E_hCySS in MAEC also provide a basis to examine whether functional changes occur in association with oxidation of the Cys in proteins associated with the cytoskeletal network. After 1 h at 0 mV, the 15 cytoskeletal proteins were 9.5% more oxidized than at -150 mV (Supplement 2). To determine whether a functional change in the cytoskeleton could be observed in association with this oxidation, we examined the cytoskeletal appearance in MAEC by fluorescence microscopy of filamentous actin (F-actin). Results showed that F-actin staining was more intense at 0 mV (1.7 fold, Figure 5A) while it showed no change in total caveolin-1 level examined for a cytoskeleton- associated protein (Figure 5B). Furthermore, treatment with cytochalasin D (+Cyto D), a chemical that disrupts actin structure by inhibiting formation of F-actin, caused a much more dramatic effect under conditions where the cytoskeletal proteins were more oxidized (Figure 5C). The data show that functional changes occur in association with oxidation of proteins in the relatively reduced cytoskeletal network.

The most oxidized 20% of cytoplasmic peptides in HT29 cells had network functions associated with cancer, cell signaling and post-translational modification. The top pathway (Figure 4B, Score 51) included the docking proteins 14-3-3 and peptides from proteins in translation initiation and elongation. Systems known to function in redox signaling, NF-κB and AMP kinase, were present as central nodes in this functional pathway analysis (Figure 4B). ANXA1, ANXA4, ATP1A1, EPCAM, FASN, G6PD, and MIP are identified molecules associated with NF-κB and AMP kinase central nodes (Figure 4B). Top canonical pathways identified included PI3K/Akt signaling ($p = 5.9 \times 10^{-05}$), Myc-mediated apoptosis signaling ($p = 3.98 \times 10^{-04}$) and 14-3-3-mediated signaling ($p = 5.4 \times 10^{-04}$). As with the association of reduced peptides with cytoskeletal networks, the association of relatively oxidized peptides with regulatory proteins of cell signaling suggests a redox organizational structure of the most oxidized Cys.

We also examined published data for MAEC [26] to determine whether proteins associated with the functional network in Figure 4B were also more oxidized. Five proteins were present, 14-3-3 (YWHAZ), large ribosomal protein 10A (RL10A), annexin A1 (ANXA1), adenyl cyclase-associated protein (CAP1) and eukaryotic translation

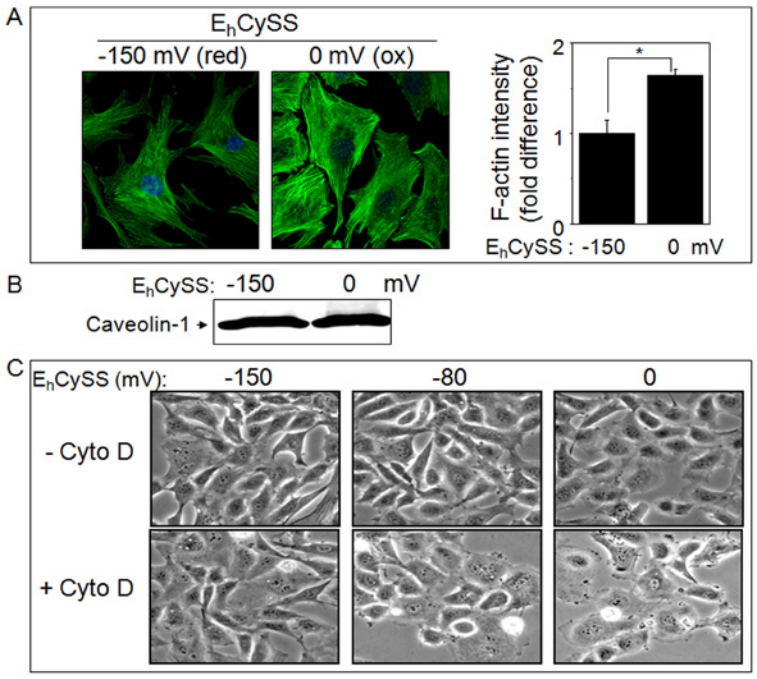

Figure 5: Elevated actin polymerization by oxidation of extracellular thiol/disulfide (cysteine/cystine) redox state (E_hCySS). A, Mouse aortic endothelial cells (MAEC) exposed to a more reduced (-150 mV) or a more oxidized (0mV) E_hCySS were examined for F-actin formation by immunofluorescence [BODIPY FL phallacidin (Molecular Probes) for labeling F-actin). Data in bar graph represents means ± SE; n = 3. B, Caveolin-1 protein amount was examined by Western blotting under the same redox condtions used for "A". C, Cytocahalasin D-induced disruption of cytoskeletal structure was elevated in a more oxidized extracellular redox environment [-150 mV (most reduced); -80 mV (average); 0 mV (most oxidized),[26]].

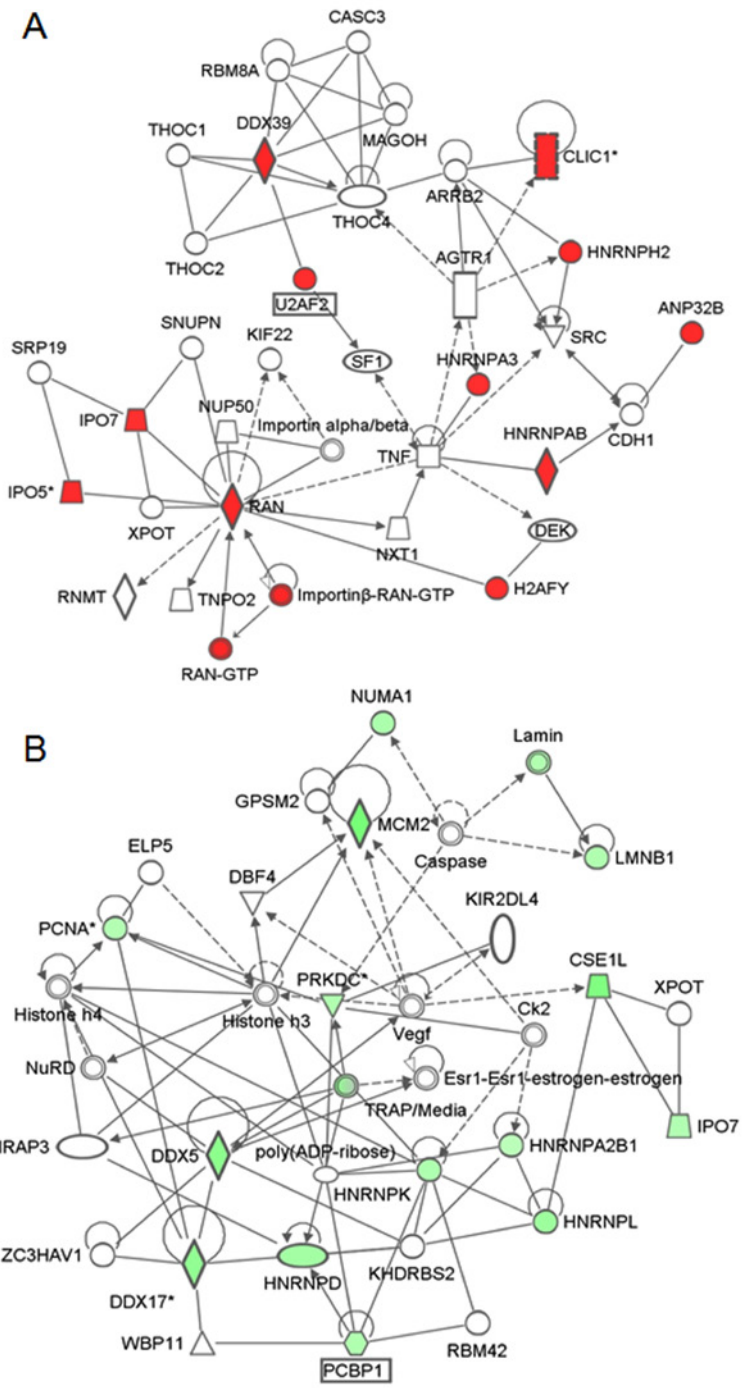

Figure 6: Association of nuclear peptidyl Cys with functional networks according to reduction and oxidation of Cys residues. Percent reduction values for the 36 most reduced Cys (Panel A) and the 36 most oxidized Cys (Panel B) were analyzed by Ingenuity Pathway Analysis. In A, the reduced nuclear peptides had network functions associated with molecular transport and RNA post-transcriptional modification (Score 26). In B, The most oxidized nuclear peptides showed functional networks associated with poly-ADP-ribose, caspase and histones H3 and H4 (Score 35). Abbreviations: ANP32B, acidic leucine-rich nuclear phosphoprotein 32 family member B; CASC3, cancer susceptibility candidate 3; CLIC1, chloride intracellular channel 1; CSE1L, chromosome segregation 1-like; DDX5, DEAD (Asp-Glu-Ala-Asp) box polypeptide 5; DDX17, DEAD box polypeptide 17; DDX39, DEAD box polypeptide 39; HNRNPA3, heterogeneous nuclear ribonucleoprotein A3; HNRNPAB, heterogeneous nuclear ribonucleoprotein AB; HNRNPA2B1, heterogeneous nuclear ribonucleoprotein A2/B1; HNRNPD, heterogeneous nuclear ribonucleoprotein D; HNRNPH2, heterogeneous nuclear ribonucleoprotein H2; HNRNPL, heterogeneous nuclear ribonucleoprotein L; H2AFY, core histone macro-H2A.1; IPO5, importin 5; IPO7, importin 7; LMNB1, lamin B1; MCM2, minichromosome maintenance complex component 2; NXT1, NTF2-like export factor 1; NUMA1, nuclear mitotic apparatus protein 1; PCBP1, poly(rC) binding protein 1; PCNA, proliferating cell nuclear antigen; PRKDC, protein kinase, DNA-activated, catalytic polypeptide; RAN, GTP-binding nuclear protein; RBM42, RNA binding motif protein 42; THOC1, THO complex 1; TRAP/Media, regulator for estrogen receptor signaling; WBP11, WW domain binding protein 11; XPOT, exportin.

elongation factor 1B2 (EEF1B2) with an average of 29% oxidation at -150 mV and 40% oxidation at 0 mV. These were not different from the average for the overall MAEC peptides, indicating that no redox response occurred due to this experimental manipulation. However, we further used IPA to analyze all 144 proteins detected in MAEC for functional network associations with extent of oxidation at 0 mV compared to -150 mV extracellular redox potential, E_hCySS. These analyses revealed 9 functional networks, two of which included PI3K/Akt (Cancer, post-translational modification, protein folding, Score 34, see Supplement 3A for network and Supplement 4 for legend) and Myc (Protein synthesis, RNA post-transcriptional modification, cancer, Score 27, see Supplement 3B for network and Supplement 4 for Legend). Thus, the data using IPA for analysis of oxidized proteins in MAEC verify the redox network findings for HT29 cells.

Pathway analysis of nuclear peptides. The smaller number of peptides obtained for nuclei and mitochondria did not allow stratification by quintiles as used for cytoplasmic peptides. For nuclei, the skewed distribution resulted in a difference between median and mean, which we arbitrarily used as a basis for comparison, selecting those more oxidized than average and comparing to an equal number of the most reduced peptides. This yielded two subsets, the most reduced 36 peptides (average reduction, 93.3%) and the 36 most oxidized peptides (average reduction, 72.4%). The reduced nuclear peptides had network functions associated with RNA post-transcriptional modification (Table 2; Score 32) and molecular transport [centered around Ran (GTPase Ran, NP_006316.1)] (Figure 6A; Score 26). Ran functions in regulation of both protein import and RNA export from nuclei, and previous research has established redox-dependent functions [53]. Sensitivity of HNRNP to oxidative stress has been widely reported [54]. Consequently, the association of more reduced proteins with Ran signaling and post-transcriptional processing suggests a redox network structure vulnerable to oxidative stress.

The most oxidized nuclear peptides showed functional networks associated with poly-ADP-ribose, caspase and histones H3 and H4 (Figure 6B; Score 35). The top canonical pathway was associated with granzyme B signaling ($p = 1.98 \times 10^{-06}$), a pathway of immune-mediated cell killing. A number of oxidized peptides were HNRNP that function in post-transcriptional processing of RNA. The results show that the most oxidized nuclear peptides associate with cell death and/or cell stress responses and differ from the nuclear network structure associated with the most reduced peptides.

Pathway analysis of mitochondrial peptides. The number of mitochondrial peptides was small (37), so analyses were performed with peptides more reduced than average (n=24) and more oxidized than average (n=13). Pathway analysis (score 39) in Figure 7A showed that more reduced peptides associated with functional networks of lipid metabolism, cancer, and small molecule biochemistry including metabolism of amino acids, citric acid cycle intermediates and glutathione conjugation. This association was contrasted by more oxidized peptides associated with cellular maintenance, which included a pathway association with Complex 3 and electron-transferring flavoproteins, the mitochondrial translation protein Tu, adenine nucleotide transporter-1 and proteins linked to insulin signaling (hexokinase-1, glutamate dehydrogenase-1, malate dehydrogenase-2) (Figure 7B; Score 36). While the number of peptides is small, the results show that redox-sensitive Cys could coordinate functions of mitochondria. Such a redox-control structure could optimize energy metabolism and render cells vulnerable to oxidants that disrupt this network structure.

The complexity of mitochondrial intermediary metabolism and energy regulation does not provide simple interpretation concerning whether these observed pathway associations should be investigated as potential network targets of oxidative stress. However, if the redox-sensitive Cys have central functions in metabolic control, then these Cys must be conserved. Searches of the Uniprot database showed that all of the redox-sensitive Cys associated with the networks except for C107 of SDHC (succinate dehydrogenase complex, subunit C) were conserved among vertebrates (Figure 8). In contrast, average amino acid conservation was considerably lower, with less than 20% of amino acids being conserved (Figure 8). Because some amino acids have similar properties, this comparison overemphasizes the extent of total variation among the proteins; none-the-less, even with consideration of amino acid similarity, the measured Cys are relatively conserved. Total conservation of Cys at specific sites among these proteins was only 71% (688 out of 966) while conservation of the measured Cys was 94% (161 out of 172). The relative conservation of the Cys residues in the peptides associated with functional networks (Figure 7) supports the interpretation that these are part of functional redox networks in mitochondria and therefore could represent network targets of oxidative stress.

Discussion

Mass spectrometry-based proteomic methods have provided substantial improvement in ability to understand the redox proteome. Sethuraman et al. [28] showed that the ICAT reagents could be used to identify specific Cys-containing peptides oxidized in response to oxidant. In their approach, oxidant-treated cells were labeled with H-ICAT and control cells were treated with L-ICAT so that the ratio provided a measure of the extent of oxidation. This approach does not show the percent oxidation under control conditions. Earlier studies showed that actin is moderately glutathionylated at Cys 374 in A431 cells under normal conditions [55]. Subsequent research by Le Moan et al. [16] showed many protein Cys are oxidized under control conditions in yeast, and similar results were obtained for *E coli* by Leichert et al. [29] and for mouse aortic endothelial cells by Go et al. [26]. Thus, the present data further support the existing literature that many proteins exist in living cells in a partially oxidized state.

The function of such oxidation has been studied in detail for a small number of proteins, especially through glutathionylation, as shown for actin [55]. The glutathionylation data are important because they show enzymatic control of Cys oxidation [52]. As previously discussed, Cys residues differ considerably in reactivity [56] and catalytically functional Cys can be predicted by their reactivity [57]. However, most Cys are not catalytically active and most are relatively non-reactive. While evidence exists to show that some protein surface Cys can function as antioxidants [49], the present finding of associations with functional networks according to percent oxidation suggests a previously unrecognized organizational structure. The experimental studies with MAEC cells further support the interpretation that such redox organizational structure is functional. Additional detailed studies of the networks identified will be required to fully understand these associations.

The present study measured steady-state oxidation of 641 peptidyl Cys in HT29 cells and showed that average oxidation was 16% in each of the compartments evaluated. Peptides containing 2 Cys, such as the active sites of thioredoxin and related proteins, were notably absent. The explanation for this is not known but could be due to more avid binding to the steptavidin column and poor recovery. The number of

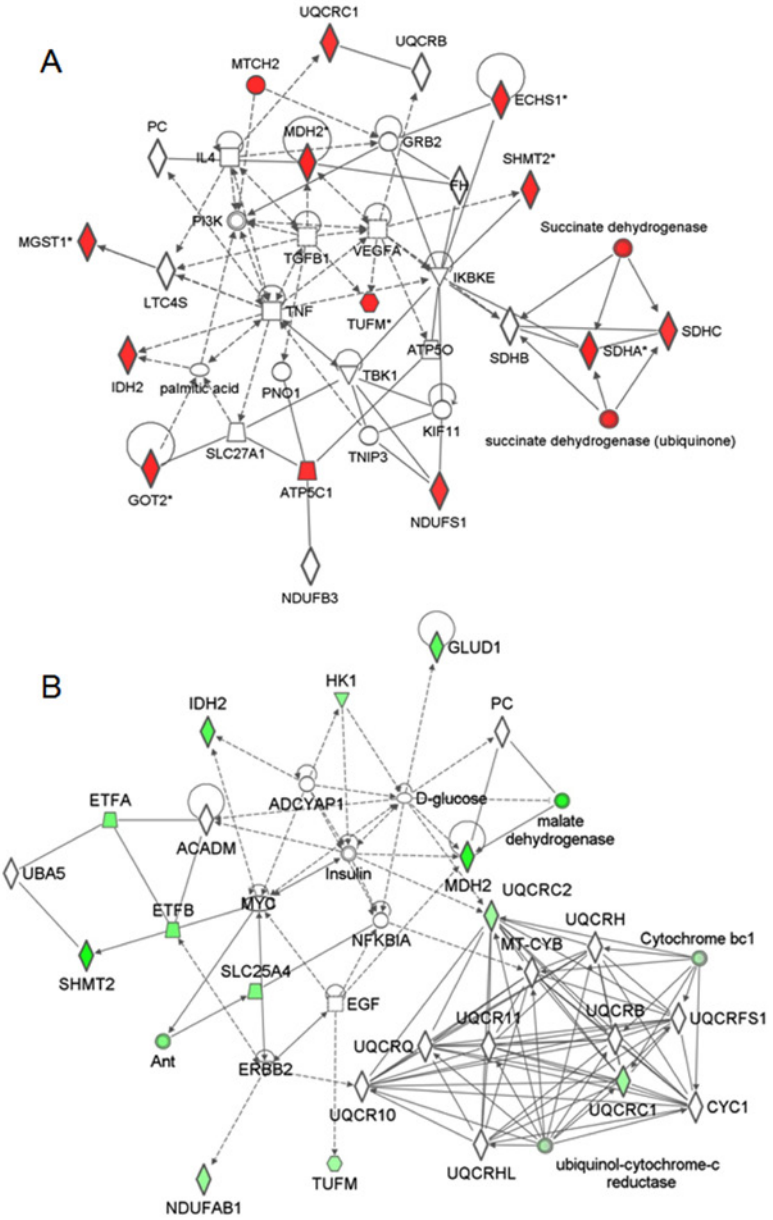

Figure 7: Association of mitochondrial peptidyl Cys with functional networks according to reduction and oxidation of Cys residues. Percent reduction values for Cys more reduced than average (Panel A) and more oxidized than average (Panel B) were analyzed by Ingenuity Pathway Analysis. In A, more reduced peptides (n=24) associated with functional networks of lipid metabolism, small molecule biochemistry and cancer (Score 39). In B, more oxidized peptides (n=13) associated with cellular function and maintenance (Score 36). Abbreviations: ADCYAP1, adenylate cyclase activating polypeptide 1; ECHS1, short-chain enoyl-CoA hydratase; ETFA, electron-transferring flavoprotein-A; ETFB, electron-transferring flavoprotein-B; GLUD1, glutamate dehydrogenase-1; GOT2, aspartate aminotransferase-2; HK1, hexokinase-1; IDH2, isocitrate dehydrogenase-2; MDH2, malate dehydrogenase-2; MGST1, microsomal glutathione-S-transferase-1; MTCH2, mitochondrial carrier homolog-2; NDUFAB1, NADH-ubiquinone oxidoreductase subunit AB1 (ACPM, acyl carrier protein, mitochondrial); SHMT2, serine hydroxymethyltransferase-2; SLC25A4, solute carrier family 25-A4 (adenine nucleotide translocase-1, ANT1); SDHA, succinate dehydrogenase subunit A; SDHC, succinate dehydrogenase subunit C; TBK1, TANK-binding kinase 1; TUFM, mitochondrial translational protein Tu; UQCRB, ubiquinol-cytochrome c reductase binding protein; UQCRC1, ubiquinol-cytochrome c reductase core protein 1; UQCRC2, ubiquinol-cytochrome c reductase core protein 2; UQCRQ, ubiquinol-cytochrome c reductase, complex III subunit VII.

Cys measured in this analysis is large compared to the number studied in previous redox compartmentation studies but still represents a small number compared to the total number of Cys encoded by the human genome. A total of nearly 2000 Cys were measured overall and showed similar results but most were not included because they were not detected in at least 3 of the 5 experiments. The discussion that follows must be considered with the limitation that even if all 2000 were included, it would only constitute 1% of the total Cys proteome. Database searches of the most reduced and most oxidized peptides measured showed that these Cys were conserved in vertebrate evolution, suggesting that the partially oxidized Cys residues have biologic functions. Pathway analysis showed that the most reduced cytoplasmic Cys associated with functional pathways of the cytoskeleton, the most reduced nuclear Cys associated with Ran

signaling and RNA post-translational processing and the most reduced mitochondrial Cys associated with energy metabolism, cell growth and proliferation. In contrast, pathway analysis showed that more oxidized cytoplasmic Cys associated with PI3/Akt signaling, Myc-mediated apoptosis signaling and 14-3-3-mediated signaling. It also showed that more oxidized nuclear Cys associated with granzyme B signaling and more oxidized mitochondrial Cys with Complex 3 and proteins that maintain cellular homeostasis, including the mitochondrial translation protein Tu, adenine nucleotide transporter-1 and proteins linked to insulin signaling (hexokinase-1, glutamate dehydrogenase-1, malate dehydrogenase-2). The association of more reduced and oxidized Cys with functional pathways suggests that compartmental redox networks represent important sites of pathway integration that could be susceptible targets in disease. More complete targeted analysis of subcellular compartments and protein complexes can be expected to improve details of these pathways.

This redox organizational structure implies that molecular adaptation within a network can compensate for modifications in discrete protein targets. If this is true, then disease mechanisms involving redox networks must be viewed in terms of effects on the functional system and not single molecular modifications. This has important implications for cancer signaling, drug development and aging. For instance, the cytoplasmic network structure for relatively oxidized peptides, including PI3/Akt, Myc and 14-3-3, has been extensively targeted in therapeutic development for cancer [58-60], yet highly targeted drugs fail to achieve desired treatment outcomes [61,62]. The present results suggest that simultaneous targeting of multiple targets within a network may be needed. This has important practical implications for efforts to target redox central nodes such as thioredoxin reductase-1 and thioredoxin-1 in cancer. Adaptive network responses could be variable due to diet, age, genetics or other factors, resulting in heterogeneity in therapeutic efficacy. Complementary targeting of other central nodes or adaptive mechanisms could provide a critical approach to overcome such therapeutic limitations.

Biochemical data show that most intracellular proteins contain peptidyl Cys predominantly as thiols and that most extracellular proteins contain peptidyl Cys predominantly as disulfide forms. These

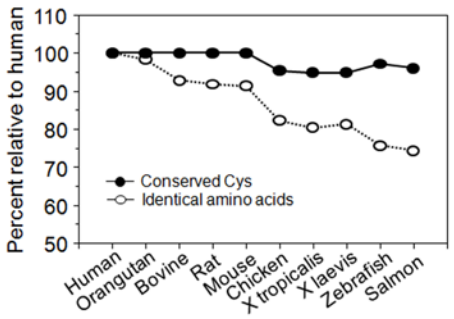

Figure 8: Evolutionary conservation among vertebrates of mitochondrial peptidyl Cys associated with functional networks. The UniProt database was searched for protein amino acid sequences of 10 vertebrate species for the peptidyl Cys associated with functional pathways in Figure 7. The species included human (*Homo sapiens*), Sumatran orangutan (*Pongo abelii*), bovine (*Bos taurus*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), chicken (*Gallus gallus*), African clawed frog (*Xenopus laevis*), Western clawed frog (*Xenopus tropicalis*), zebrafish (*Danio rerio*) and Atlantic salmon (*Salmo salar*). The percentage of identity relative to humans was calculated for each of the Cys (filled circles), and the percentage of amino acid identity relative to humans was obtained from a BLAST search in Uniprot (open circles).

data have been interpreted to mean that cellular proteins contain only thiols unless specific oxidative mechanisms are present to introduce oxidized forms [18]. However, early measurements of cellular GSH/GSSG showed that cellular NADPH-dependent reduction systems do not maintain a fully reduced state [33]. While the redox potential of the NADPH/NADP couple is maintained at about -400 mV, the redox potential of GSH/GSSG is about -250 mV, i.e., the concentrations of individual components are orders of magnitude away from equilibrium values [32]. Measurements of the Trx systems similarly show that these central reductants are not in equilibrium with the NADPH/NADP couple [15], and studies of redox pathways from NADPH → thioredoxin reductase-1 → Trx1 → redox factor-1 → p50 (NF-κB) as well as NADPH → thioredoxin reductase-2 → Trx2 → peroxiredoxin-3 show that redox components of protein thiol pathways are not in equilibrium [1]. Thus, the results of the present study showing that peptidyl Cys oxidation under basal conditions is 16% is consistent with these earlier findings.

Research on redox signaling mechanisms has led to the recognition that a subset of peptidyl Cys function as redox switches [4,63] and exist in reversibly modified forms [1]. The number of Cys residues shown to function in redox signaling is relatively small, but studies of protein glutathionylation [5], sulfenylation [7] and nitrosylation [6], indicate that dozens to hundreds of proteins could have such functions. Mouse aortic endothelial cells respond to oxidized extracellular cystine redox potential with oxidation of cellular proteins [26]. Oxidation of Cys residues of 34 cytoskeletal and actin-associated proteins [26] indicates that a subset of peptidyl Cys provides a regulatory structure to integrate cell signaling and control [17]. This subset of "redox-sensing Cys" appears to be distinct from "redox-signaling Cys", providing a regulatory structure in which a subset of Cys residues functions orthogonally to Cys residues in signaling and metabolic pathways [17]. Such a regulatory structure would include responsiveness to NO, as well as other modifications not discriminated by the current approach. The present data show that different Cys within the same protein (e.g., IDH2, Figure 7A, 7B) have different percent reduction i.e., 92.0% and 70.6%, supporting the interpretation that individual proteins can have multiple redox sensors. Keap-1, the sensor for activation of the antioxidant response element by Nrf2, has previously been shown to have multiple redox sensors [64]. Such a regulatory structure has the attractive characteristic that it provides a molecular economy in regulation and integration of diverse systems.

The present observations support a need for the application of systems biology methods to understand disease mechanisms involving the Cys proteome. Systems biology studies show that certain network structures have inherent stability. For instance, a hierarchical, scale-free network structure of metabolism is relatively stable to environmental changes [65]. Organization of redox systems in such a structure could account for features of redox biology and "cross-talk" of biologic systems. Importantly, such a redox network structure could allow adaptation to site-specific toxic effects, thereby protecting against system failure until conditions are achieved that cause a catastrophic collapse.

The present study was designed to provide enough biological replicates of a single experimental condition for cultured HT29 cells for a critical evaluation of the differences in oxidation of Cys residues among proteins with different subcellular localizations. This design did not provide the expected results in patterns of compartmental protein oxidation. In terms of the applicability of the findings to other cell types,

the HT29 cells are a colon cancer cell line and this makes it likely that some of the current results may differ from what one might find in non-transformed cells. Transformed cells typically have altered pathways of cell signaling and homeostasis of energy metabolism and cytoskeleton. Although additional functional data from mouse aortic endothelial cells and mouse liver examination support the findings from HT29 studies, the general applicability of the specific pathway results may be limited. None-the-less, the observed redox network organizational structure may be critical for future research designs for redox signaling. The percent oxidation data support earlier findings of partial oxidation of proteins in cells [16] and emphasize that definition of protein redox states in the control state may be critical for interpretation of results of experimental manipulations. Additionally, the existence of a redox network structure suggests that network responses are more robust than measurement of single proteins. Network response characteristics therefore may provide better means to evaluate redox signaling than targeted analyses of specific proteins. A need to develop databases to support research on redox network structures was recently discussed [66]. Assembly of available knowledge on redox-regulated Cys and Met residues in specific proteins into a user-friendly database structure could be especially useful for research into the complex interactions among signaling pathways and subcellular compartments.

In summary, the present research shows that Cys residues of proteins exist in a partially oxidized state and associate with functional networks according to the percent oxidation. Results show that average steady-state oxidation of Cys residues is similar for cytoplasmic, nuclear and mitochondrial proteins. Within compartments, more oxidized and more reduced Cys associate with functional networks, showing that a steady-state redox organizational structure exists for the Cys proteome. Examination of measured Cys showed that they are conserved in vertebrate evolution, implying essential function. The most reduced cytoplasmic Cys associated with cytoskeletal proteins while the most oxidized associated with proteins of signaling pathways. The results provide a basis for crosstalk between signaling pathways and show that redox signaling must be considered within a network structure rather than as isolated events within insular pathways or subcellular compartments. A critical significance of this work is that therapeutic agents to impact health through thiol redox mechanisms must be appropriately designed for redox network targeting or they are likely to be ineffective.

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