Identification of DUOX1-dependent redox signaling through protein S-glutathionylation in airway epithelial cells

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The NADPH oxidase homolog dual oxidase 1 (DUOX1) plays an important role in innate airway epithelial responses to infection or injury, but the precise molecular mechanisms are incompletely understood and the cellular redox-sensitive targets for DUOX1-derived H2O2 have not been identified. The aim of the present study was to survey the involvement of DUOX1 in cellular redox signaling by protein S-glutathionylation, a major mode of reversible redox signaling. Using human airway epithelial H292 cells and stable transfection with DUOX1-targeted shRNA as well as primary tracheal epithelial cells from either wild-type or DUOX1-deficient mice, DUOX1 was found to be critical in ATP-stimulated transient production of H2O2 and increased protein S-glutathionylation. Using cell pre-labeling with biotin-tagged GSH and analysis of avidin-purified proteins by global proteomics, 61 S-glutathionylated proteins were identified in ATP-stimulated cells compared to 19 in untreated cells. Based on a previously established role of DUOX1 in cell migration, various redox-sensitive proteins with established roles in cytoskeletal dynamics and/or cell migration were evaluated for S-glutathionylation, indicating a critical role for DUOX1 in ATP-stimulated S-glutathionylation of β-actin, peroxiredoxin 1, the non-receptor tyrosine kinase Src, and MAPK phosphatase 1. Overall, our studies demonstrate the importance of DUOX1 in epithelial redox signaling through reversible S-glutathionylation of a range of proteins, including proteins involved in cytoskeletal regulation and MAPK signaling pathways involved in cell migration.

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Introduction

The respiratory epithelium forms a first line of pulmonary defense against inhaled microorganisms, allergens and other pollutants, by creating tightly controlled physical barrier that minimizes microbial invasion and exposure of critical lung constituents to noxious environmental agents, and by evoking innate responses to diverse pathogen- or damage-associated molecular patterns to initiate host defense mechanisms and/or wound responses to infection or injury. One aspect of such innate responses is the apical production of hydrogen peroxide (H2O2) in response to various stimuli, originating primarily from two recently identified NADPH oxidase homologs, the dual oxizides 1 and 2 (DUOX1/2). This regulated H2O2 production is believed to represent an important component of oxidative antimicrobial surveillance at mucosal surfaces [1–4], although the relative roles of DUOX1 or DUOX2 in such mucosal host defense are still somewhat unclear [5]. In addition to its proposed involvement in apical oxidative host defense, epithelial DUOX reportedly also participates in autocrine or paracrine cell signaling mechanisms that regulate cellular pro-inflammatory and wound responses [6–9]. As such, DUOX has been demonstrated to control the activation of Src family kinases and epidermal growth factor receptors (EGFR), resulting in downstream cell signaling via extracellular signal-regulated kinase (ERK) and/or nuclear factor (NF)-κB [5,10].

Activation of DUOX in response to epithelial infection or injury often involves the initial secretion of cellular damage signals such as ATP, which promotes Ca2+-dependent DUOX activation by...
stimulation of purinergic P2Y receptors on the epithelial surface [10]. Within the respiratory tract, such ATP-dependent wound responses primarily rely on the major constitutive airway epithelial isoform, DUOX1, and our recent studies have demonstrated a key role for DUOX1 in ATP-dependent epithelial wound responses and epithelial regeneration [9,11,12], analogous to observed DUOX-dependent injury responses in zebrafish or Drosohila [6,8]. Nevertheless, in spite of reports indicating DUOX-dependent cell signaling through oxidation of selective cysteines within e.g. Src family kinases [8,11] or ADAM-family sheddases [11,13,14], no studies exist that systematically evaluate the cellular targets that are subject to reduct-dependent regulation by activation of DUOX1. Moreover, while some studies suggest that DUOX1-mediated signaling involves selective oxidation of redox-sensitive targets in e.g. signalsomes (e.g. [11,15]), others imply that DUOX activation generates H2O2 gradients capable of paracrine oxidative signaling over several cell distances [7,16].

One important mode of oxidant-dependent redox signaling involves the reversible oxidation of selected protein cysteine residues, and one major consequence of such cysteine oxidation is the formation of a mixed disulfide with GSH, the most abundant cellular low-molecular weight thiol, by a process known as S-glutathionylation. Protein S-glutathionylation may be a critical event in reversal of cysteine oxidation, but may also actively control enzymatic activity, protein–protein interactions, or protein turnover [17,18]. A potential role for S-glutathionylation in e.g. epithelial wound responses is indicated by observations of increased overall S-glutathionylation at the wound margin of injured epithelial monolayers [19] and impaired cell migration in endothelial cells that overexpress glutaredoxin-1, a major enzyme involved in reversing S-glutathionylation [20]. Moreover, S-glutathionylation of several specific proteins has been associated with alterations in cell migration dynamics [21–23], although the precise molecular consequences of protein S-glutathionylation and the proximal mechanisms that promote S-glutathionylation are largely unknown. The present studies were conducted to determine the contribution of DUOX1 in protein S-glutathionylation in the context of ATP-mediated epithelial wound responses, and to identify protein targets for DUOX1-dependent S-glutathionylation. Our studies reveal the importance of DUOX1 in ATP-stimulated S-glutathionylation of a diverse number of proteins, including several key proteins involved in cytoskeletal control, stress responses, and cellular signaling pathways involved in cell migration, and thereby provide additional insights into the mechanistic aspects of DUOX1-mediated epithelial wound responses.

Experimental

Cell culture and treatments

Experiments were performed with a human pulmonary mucoepidermoid carcinoma cell line NCI-H292 (ATCC), which was maintained in RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C and 5% CO2. To address the role of DUOX1, a stable H292 cell line was generated that was transfected with pRNATin-H1.2/Hygro vector (GenScript, Piscataway, NJ) containing a DUOX1-targeted shRNA sequence (H292-shDUOX1) as well as a corresponding control cell line containing empty vector (H292-CTL). Compared to corresponding controls, H292-shDUOX1 cells are almost completely deficient in DUOX1 mRNA and protein [11]. Additional studies were performed with primary mouse tracheal epithelial (MTE) cells, which were isolated from the tracheas of C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) using overnight incubation with 0.1% protease 14 (Sigma–Aldrich, St. Louis, MO) and cultured on rat tail collagen I gel (BD Biosciences, San Jose, CA) in DMEM/F12 media (Invitrogen, Grand Island, NY) supplemented with 20 ng/ml cholera toxin (List Biological Laboratories, Campbell, CA), 10 ng/ml EGF (Calbiochem, San Diego, CA), 5 μg/ml insulin (Sigma), 5 μg/ml transferrin (Sigma), 100 nM dexamethasone (Sigma), 15 μg/ml bovine pituitary extract (Invitrogen), 2 mM L-glutamine, and 50 U/50 μg/ml Penicillin/Streptomycin (Pen/Strep) (Invitrogen), as described previously [24,25]. Similarly, MTE cells were isolated from DUOX1 knockout mice that were originally generated using a retroviral-based gene-trapping method and obtained from Lexicon Pharmaceuticals, Inc. (The Woodlands, TX, USA), and backcrossed onto a C57BL/6J background [26]. Genotypic of DUOX1 knockout mice was performed as described previously [26]. MTE cells were used at passages 2–4.

For experimentation, H292 or MTE cells were seeded at 1 × 105 cells/cm2 in 24-well plates (BD Labware, Bedford, MA) and cultured for an additional 3–4 days. Prior to cell stimulation, cells were starved overnight in serum-free medium (H292) or EGF-lacking medium (MTE) and stimulated with exogenous ATP (Sigma), and media or cell lysates were collected for the various analyses described below.

Analysis of cellular H2O2 production

For analysis of extracellular H2O2 production, H292 or MTE cells were seeded on 24 plates and cultured to full confluence (~150,000 cells/well), and after replacing media with 200 μL HBSS, cells were stimulated with ATP and conditioned media was removed at indicated times and mixed with 10 μg/ml lactoperoxidase (Sigma) and 1 mM tyrosine for 15 min, and resulting dityrosine production was analyzed by HPLC as a measure of H2O2 production [11]. Alternatively, H2O2 in conditioned media was analyzed using the Amplex Red assay (Invitrogen) according to the manufacturer’s instructions. Specificity for H2O2 was verified by sample pretreatment with catalase (2000 U/ml; Sigma), and H2O2 production was calculated using similar analysis of exogenous standards of H2O2 in HBSS, and expressed as nmol/106 cells. Cellular production of reactive oxygen species was determined 15-min pre-loading of H292 cells or MTE cells in chamber slides with 10 μM 2,7’-dichlorodihydrofluorescein diacetate (H2DCF-DA), and visualization of DCF fluorescence as an indicator of cellular oxidant production in response to cell stimulation with ATP, using a Nikon Eclipse E800 fluorescence microscope.

Analysis of cell migration

H292 cells or MTE cells were seeded on fibronectin-coated polycarbonate tissue culture inserts (8-μm pore-size; NUNC) at 1 × 105 cells/well, and incubated for 6 h, after which media with non-attached cells was removed, and cells were further incubated in the absence or presence of ATP for an additional 24 h for analysis of epithelial cell migration by haplomaxis, as previously described [9].

Quantitative analysis of protein S-glutathionylation

Protein S-glutathionylation was determined as previously described [27], with some modifications. Briefly, stimulated or unstimulated H292 or MTE cells were lysed in RIPA buffer containing of 50 mM N-ethyl maleimide, and cell lysates containing 1 mg protein were precipitated with TCA (6% final concentration), and protein pellets were washed 3x with 1.5% TCA and resuspended in 1 ml of 0.2 M potassium phosphate buffer containing 1 mM EDTA. Aliquots (300 μl) of this final protein solution were adjusted to pH 8.2–8.4 with NaOH and mixed with DTT
(7 mM final concentration) to reduce PSSG to GSH, which was subsequently derivatized for HPLC analysis by mixing (1:1) with 40 mM monobromobimane (mBrB; Calbiochem) in CH$_3$CN for 15 min. After protein precipitation with TCA (5%), GSH-mBrB was quantified by HPLC with fluorescence detection, as described previously [28]. Control samples were processed similarly without addition of DTT. GSH concentrations were determined using exogenous standards, and PSSG was expressed as nmol/mg protein.

**Analysis of protein S-glutathionylation after cell loading with BioGEE**

Biotinylated glutathione ethyl ester (BioGEE) was prepared by reacting 0.5 M glutathione ethyl ester (Sigma) with 0.5 M EZ-link sulfo-NHS-biotin (Pierce) in 50 mM NaHCO$_3$ (pH 8.5) as described previously [29], and was added to H292 cells or MTE cells at a final concentration of 250 μM for 1 h, prior to cell treatment with or without ATP for 10 min. After treatment, cells were placed on ice and washed with cold PBS containing 50 mM N-ethylmaleimide (NEM) to remove unreacted BioGEE and lysed in 1.5 ml RIPA buffer containing 50 mM NEM. Cell lysates were mixed 1:1 with nonreducing sample buffer for separation by SDS-PAGE, transferred to PVDF membranes and blotted with streptavidin-HRP to detect biotin-labeled proteins. For identification of biotinylated proteins by Western blot, cleared cell lysates were centrifuged on G25 columns to remove excess biotinylating agent, and biotinylated proteins were collected with high capacity neutravidin beads (Pierce) by constant rotation overnight at 4°C. Neutravidin beads were washed 4x with RIPA buffer and 2x with PBS containing 1% SDS, after which biotinylated proteins were eluted from the beads by 30 min incubation with PBS containing 1% SDS and 10 mM DTT at room temperature. Eluted protein and corresponding whole cell lysates (as input controls) were mixed with reducing sample buffer for analysis by SDS-PAGE, transferred to PVDF, and blotted with using antibodies against β-actin (Cell Signaling), peroxiredoxin 1 (Prx1; Abcam), c-Src (L4A1; Cell Signaling), and MAPK phosphatase 1 (MKP-1; Santa Cruz). Primarily antibodies were probed with rabbit or mouse-specific secondary antibodies conjugated with HRP (Cell Signaling) and detected by enhanced chemiluminescence (Pierce).

**Identification of S-glutathionylated proteins by LC–MS/MS**

Confluent H292 cells in 100 mm dishes were preloaded with BioGEE and stimulated with ATP (100 μM) as described above, and cell lysates containing 2.5 mg protein were applied to G25 columns to remove unreacted BioGEE and high-capacity neutravidin beads (250 μl; Pierce) by constant rotation overnight at 4°C. Neutravidin beads were washed 4x with RIPA buffer and 2x with PBS containing 1% SDS, after which biotinylated proteins were eluted from the beads by 30 min incubation with PBS containing 1% SDS and 10 mM DTT at room temperature. Eluted protein and corresponding whole cell lysates (as input controls) were mixed with reducing sample buffer for analysis by SDS-PAGE, transferred to PVDF, and blotted with using antibodies against β-actin (Cell Signaling), peroxiredoxin 1 (Prx1; Abcam), c-Src (L4A1; Cell Signaling), and MAPK phosphatase 1 (MKP-1; Santa Cruz). Primarily antibodies were probed with rabbit or mouse-specific secondary antibodies conjugated with HRP (Cell Signaling) and detected by enhanced chemiluminescence (Pierce).

Quantitative data are presented as mean ± S.E. and statistical differences were determined using Student’s t-test and differences were considered significant at $p < 0.05$.

**Results**

**DUOX1 activation mediates ATP-dependent H$_2$O$_2$ production and protein S-glutathionylation**

We first confirmed the importance of DUOX1 in cellular H$_2$O$_2$ production response to extracellular ATP, using two separate epithelial cell models, namely human airway epithelial H292 cells and mouse tracheal epithelial (MTE) cells. As shown in Fig. 1A, stimulation of H292 cells with ATP induced rapid and transient...
| Protein name | Accession number | M.W. (kDa) | No. of peptides |
|--------------|------------------|------------|----------------|
| **Table 1**  |                  |            |                |
| **Proteins identified in unstimulated (CNTL) and ATP stimulated H292 cells.** | | | |
| **1** Cluster of Uncharacterized protein (IPI00022434) | | | |
| 1.1 Uncharacterized protein | IPI00022434 | 72 | 8 |
| 1.2 Uncharacterized protein | IPI00878517 | 56 | 6 |
| **2** Cluster of Isoform DPI of Desmoplakin (IPI00013933) | | | |
| 2.1 Isoform DPI of Desmoplakin | IPI00013933 | 332 | 12 |
| 2.2 Desmoplakin Ia | IPI00969616 | 279 | 10 |
| 2.3 cDNA FLJ63543, highly similar to Desmoplakin | IPI01009332 | 156 | 9 |
| 2.4 cDNA FLJ26719 fis, clone PNC03379 | IPI00746877 | 20 | 0 |
| **3** Desmoglein-1 | | | |
| 3.1 Uncharacterized protein IPI00645534 | IPI00025753 | 114 | 5 |
| 3.2 Uncharacterized protein IPI00917820 | IPI00025753 | 114 | 5 |
| **4** Cluster of actin, cytoplasmic 1 (IPI00021439) | | | |
| 4.1 Actin, cytoplasmic 1 | IPI00352883 (+1) | 42 | 4 |
| 4.2 Isoform 1 of POTE ankyrin domain family member E | IPI00479743 | 121 | 2 |
| 4.3 Putative beta-actin-like protein 3 | IPI00745539 | 121 | 2 |
| 4.4 POTE ankyrin domain family member I | IPI00969616 | 279 | 10 |
| 4.5 cDNA FLJ52761, highly similar to Actin, aortic smooth muscle | IPI09848797 | 37 | 2 |
| 4.6 POTE ankyrin domain family member J | IPI00021428 | 42 | 3 |
| 4.7 Desmoglein-1 | IPI00746877 | 20 | 0 |
| 4.8 Isoform 1 of POTE ankyrin domain family member E | IPI00025753 | 114 | 5 |
| **5** Cluster of Heat shock protein HSP 90-beta (IPI000414676) | | | |
| 5.1 Heat shock protein HSP 90-beta | IPI00382470 (+1) | 98 | 0 |
| 5.2 Heat shock protein 90Bb | IPI00555565 | 58 | 2 |
| 5.3 Isoform 2 of Heat shock protein HSP 90-alpha | IPI00021290 | 121 | 5 |
| 5.4 Putative heat shock protein HSP 90-beta 4 | IPI00746877 | 20 | 0 |
| 5.5 Putative heat shock protein HSP 90-alpha A2 | IPI00025753 | 114 | 5 |
| 5.6 Uncharacterized protein | IPI00878517 | 56 | 6 |
| 5.7 Uncharacterized protein | IPI00878517 | 56 | 6 |
| **6** Junction plakoglobin | | | |
| 6.1 Isoform 2 of Filamin-A (IPI000302592) | | | |
| 7.1 Isoform 2 of Filamin-A | IPI00302592 (+2) | 280 | 0 |
| 7.2 Protein | IPI00893150 | 10 | 0 |
| 7.3 Filamin A, alpha | IPI01018650 | 25 | 0 |
| **8** Cluster of cDNA FLJ66442, highly similar to ATP-citrate synthase (IPI00394838) | | | |
| 8.1 cDNA FLJ66442, highly similar to ATP-citrate synthase | IPI00382470 (+1) | 98 | 0 |
| 8.2 ATP-citrate synthase | IPI00021290 | 121 | 5 |
| **9** Hornerin | | | |
| 9.1 Cluster of Glyceraldehyde-3-phosphate dehydrogenase (IPI000219018) | | | |
| 10.1 Glyceraldehyde-3-phosphate dehydrogenase | IPI00219018 | 36 | 3 |
| 10.2 Glyceraldehyde-3-phosphate dehydrogenase | IPI00795257 | 32 | 3 |
| 10.3 Glyceraldehyde-3-phosphate dehydrogenase | IPI00789134 | 28 | 2 |
| **11** Cluster of Isoform 1 of Myosin-9 (IPI00019502) | | | |
| 11.1 Isoform 1 of Myosin-9 | IPI00917820 | 16 | 2 |
| 11.2 FLJ00279 protein (Fragment) | IPI00025753 | 114 | 5 |
| 11.3 Uncharacterized protein | IPI00878517 | 56 | 6 |
| **12** Cluster of Isoform 2 of Annexin A2 (IPI000418169) | | | |
| 12.1 Isoform 2 of Annexin A2 | IPI00481369 | 40 | 4 |
| 12.2 Putative annexin A2-like protein | IPI00334627 | 39 | 3 |
| 12.3 cDNA FLJ4687 fis, clone MESAN2000620, highly similar to Annexin A2 | IPI00903334 | 21 | 2 |
| **13** Filaggrin-2 | | | |
| 13.1 Uncharacterized protein | IPI00302592 (+2) | 280 | 0 |
| 13.2 Putative beta-actin-like protein 3 | IPI00746877 | 20 | 0 |
| 13.3 Similar to Kappa-actin | IPI00021428 | 42 | 3 |
| **14** Cluster of 88 kDa protein (IPI010266194) | | | |
| 14.1 88 kDa protein | IPI010266194 | 88 | 0 |
| 14.2 Uncharacterized protein | IPI00645452 (+4) | 48 | 0 |
| 14.3 Similar to Tubulin beta-2A chain | IPI00975573 | 24 | 0 |
| 14.4 TUB86 protein | IPI00646779 | 50 | 0 |
| 14.5 Tubulin beta-8 chain B | IPI00174849 | 50 | 0 |
| 14.6 Tubulin beta-4 chain | IPI00233989 | 50 | 0 |
| 14.7 Putative uncharacterized protein (Fragment) | IPI00878517 | 56 | 6 |
| 14.8 Tubulin beta 2C (Fragment) | IPI00956734 | 10 | 0 |
| 14.9 Putative tubulin beta-4q chain | IPI00018511 | 48 | 0 |
| **15** Cluster of Hemoglobin subunit beta (IPI000654755) | | | |
| 15.1 Hemoglobin subunit beta | IPI00654755 (+1) | 16 | 0 |
| 15.2 HbBm fused globin protein (Fragment) | IPI00930351 | 11 | 0 |
| 15.3 Hemoglobin subunit gamma-2 | IPI00554676 | 16 | 0 |
| 15.4 Hemoglobin subunit epsilon | IPI00217471 | 16 | 0 |
| **16** Dermcidin | | | |
| 16.1 Uncharacterized protein | IPI00027547 | 11 | 3 |
| Protein name                                                                 | Accession number | M.W. (kDa) | No. of peptides |
|------------------------------------------------------------------------------|------------------|------------|----------------|
| **Continued**                                                                 |                  |            |                |
| **17** Cluster of Uncharacterized protein (IPI00105738)                      |                  |            |                |
| 17.1 Uncharacterized protein                                                 | IPI01015738      | 80         | 0              | 3              |
| 17.2 alpha-actinin-1 isoform a                                               | IPI00739776      | 106        | 0              | 4              |
| 17.3 cDNA FLJ54718, highly similar to Alpha-actinin-1                        | IPI01025172      | 30         | 0              | 0              |
| 17.4 34 kDa protein                                                          | IPI01009456      | 34         | 0              | 2              |
| 17.5 Protein                                                                 | IPI01026210      | 29         | 2              |                |
| **18** Cluster of 268 kDa protein (IPI00942045)                              |                  |            |                |
| 18.1 268 kDa protein                                                         | IPI00942045      | 268        | 0              | 7              |
| 18.2 Uncharacterized protein                                                 | IPI01044777      | 120        | 0              | 5              |
| 18.3 Isoform 4 of Acetyl-CoA carboxylase 1                                   | IPI00396015      | 270        | 0              | 7              |
| **19** Cluster of Trifunctional enzyme subunit alpha, mitochondrial (IPI00031522) |                  |            |                |
| 19.1 Trifunctional enzyme subunit alpha, mitochondrial                      | IPI00031522      | 83         | 2              | 5              |
| 19.2 cDNA FLJ52806, highly similar to Trifunctional enzyme subunit alpha, mitochondrial | IPI00980835 | 28 | 0 | 0 |
| **20** Cluster of Pyruvate carboxylase, mitochondrial (IPI000299402)         |                  |            |                |
| 20.1 Pyruvate carboxylase, mitochondrial                                      | IPI00299402      | 130        | 0              | 5              |
| 20.2 Uncharacterized protein                                                 | IPI00975989      | 33         | 0              | 5              |
| **21** Methylcrotonoyl-CoA carboxylase subunit alpha, mitochondrial          |                  |            |                |
| 21.1 Methylcrotonoyl-CoA carboxylase subunit alpha, mitochondrial            | IPI00025807      | 273        | 0              | 2              |
| **22** Cluster of Uncharacterized protein (IPI00922694)                      |                  |            |                |
| 22.1 Uncharacterized protein                                                 | IPI00922694      | 70         | 0              | 4              |
| 22.2 Stress-70 protein, mitochondrial                                        | IPI00007765 (+1) | 74a | 0 | 4 |
| **23** Cluster of Annexin A1 (IPI00218918)                                   |                  |            |                |
| 23.1 Annexin A1                                                              | IPI00218918      | 39         | 0              | 5              |
| 23.2 Uncharacterized protein                                                 | IPI00549413      | 23         | 0              | 0              |
| **24** Cluster of Uncharacterized protein (IPI000297779)                     |                  |            |                |
| 24.1 T-complex protein 1 subunit beta                                       | IPI000297779     | 57         | 0              | 2              |
| 24.2 T-complex protein 1 subunit beta isoform 2                              | IPI00981169      | 53         | 0              | 0              |
| **25** 30 kDa protein                                                        |                  |            |                |
| 25.1 30 kDa protein                                                          | IPI01009456      | 124        | 0              | 3              |
| **26** Fatty acid synthase                                                   |                  |            |                |
| 26.1 Fatty acid synthase                                                     | IPI00024800      | 80         | 0              | 4              |
| **27** Cluster of Protein (IPI001012384)                                     |                  |            |                |
| 27.1 Protein                                                                 | IPI01012384      | 124        | 0              | 3              |
| 27.2 Importin 5                                                              | IPI00514205      | 14         | 0              | 0              |
| 27.3 Uncharacterized protein                                                 | IPI00947399      | 14         | 0              | 0              |
| **28** Isoform 2 of Plakophilin-1                                            |                  |            |                |
| 28.1 Isoform 2 of Plakophilin-1                                              | IPI00071509 (+1) | 83 | 2 | 2 |
| **29** Cluster of Protein (IPI00029806)                                      |                  |            |                |
| 29.1 T-complex protein 1 subunit beta                                       | IPI00029806      | 57         | 0              | 2              |
| 29.2 T-complex protein 1 subunit beta isoform 2                              | IPI00981169      | 53         | 0              | 0              |
| **30** Cluster of Puromycin-sensitive aminopeptidase (IPI00026216)           |                  |            |                |
| 30.1 Puromycin-sensitive aminopeptidase                                      | IPI00026216      | 103        | 2              | 2              |
| 30.2 Protein                                                                 | IPI00979097      | 49         | 0              | 0              |
| 30.3 Uncharacterized protein                                                 | IPI00979660      | 22         | 0              | 0              |
| 30.4 Uncharacterized protein                                                 | IPI00984113      | 20         | 0              | 0              |
| **31** Isoform 2 of Propionyl-CoA carboxylase alpha chain, mitochondrial      |                  |            |                |
| 31.1 Isoform 2 of Propionyl-CoA carboxylase alpha chain, mitochondrial       | IPI01009407      | 57         | 0              | 2              |
| 31.2 Uncharacterized protein                                                 | IPI00964079      | 57         | 0              | 2              |
| **32** Cluster of Elongation factor 2 (IPI001012384)                         |                  |            |                |
| 32.1 Elongation factor 2                                                      | IPI00186290      | 95         | 0              | 2              |
| 32.2 Uncharacterized protein                                                 | IPI00108586      | 65         | 0              | 0              |

Table 1 (continued)
increase in extracellular H$_2$O$_2$ production, which was maximal at 10–15 min and gradually declined thereafter, indicating transient activation of DUOX1 by ATP-dependent purinoreceptor stimulation. Moreover, ATP-induced extracellular H$_2$O$_2$ production was dramatically attenuated in H292 cells lacking DUOX1 due to transfection with DUOX1-targeted shRNA (H292-shDUOX1) [11] (Fig. 1B). Equivalent findings were obtained using the Amplex Red assay (results not shown). Complementary studies with primary MTE cells from wild-type C57BL/6j mice similarly showed increased H$_2$O$_2$ production in response to ATP stimulation, albeit at markedly lower levels compared to similar stimulation of H292 cells, and this also requires DUOX1 since it was not observed using MTE cells obtained from DUOX1 knockout mice [26] (Fig. 1C). Comparative analysis of cellular oxidant production in response to ATP, using cell preloading with the oxidant-sensitive probe H$_2$DCF, similarly indicated markedly increased oxidant production in ATP-stimulated cells from wild-type mice compared to DUOX1-knockout mice (Fig. 1D).

To determine whether ATP-dependent DUOX1 activation promotes protein S-glutathionylation as a mechanism of redox signaling, we quantified the overall cellular levels of S-glutathionylated proteins (PSSG) in response to ATP stimulation. As shown in Fig. 2A, ATP stimulation of H292 cells caused a rapid increase in overall PSSG levels, which reached an optimum 10–15 min after ATP stimulation and decreased at later time points, kinetics resembling transient DUOX1 activation (Fig. 1A) and suggesting the importance of DUOX1 in PSSG formation. Accordingly, no increases in overall protein S-glutathionylation were not associated with significant changes in overall GSH redox status, determined by analysis of GSH/GSSG ratios [30] (results not shown), indicating that DUOX1-dependent protein S-glutathionylation was most likely induced by initial oxidation of target protein cysteines rather than protein S-glutathionylation via initial formation of GSSG.

Identification of S-glutathionylated proteins by LC–MS/MS

As an alternative approach to demonstrate protein S-glutathionylation, a means to identify protein targets for glutathionylation,
H292 cells or MTE cells were pre-loaded with BioGEE prior to cell stimulation with ATP, after which protein S-glutathionylation was assessed by incorporation of biotin. Analysis of H292 cell lysates by non-reducing SDS-PAGE and streptavidin blotting revealed that ATP stimulation resulted in increased biotinylation of a number of proteins, whereas minimal increases in biotin incorporation were observed in similarly stimulated H292-shDUOX1 cells (Fig. 3A), confirming the involvement of DUOX1 in ATP-stimulated protein S-glutathionylation. Similar analysis of protein S-glutathionylation in BioGEE-loaded MTE cells from wild-type mice and DUOX1-deficient mice yielded comparable results (Fig. 3B), illustrating a critical role for DUOX1 in enhanced S-glutathionylation of a variety of proteins in response to cell stimulation with exogenous ATP.

To identify protein targets for S-glutathionylation in response to ATP stimulation, biotinylated proteins from BioGEE-loaded H292 cells were purified with avidin chromatography and separated by 1-dimensional SDS-PAGE, after which each gel lane was cut into 20 sections for in-gel protein digestion with trypsin for analysis by LC-MS/MS. Selection criteria for positive protein identification included representation by at least 2 unique peptides.
peptides, and keratins were excluded from the data set. As illustrated in Table 1, 19 S-glutathionylated proteins were identified in unstimulated H292 cells, and this number increased to 61 in ATP-stimulated cells. Sequence information for the proteins identified in control and ATP-stimulated samples is presented in Supplementary Tables S1 and S2. A number of S-glutathionylated proteins (44) were detected only in ATP-stimulated H292 cells but not in controls, indicating that these proteins are S-glutathionylated in response to ATP stimulation. In addition, among the S-glutathionylated proteins that were identified in both unstimulated and ATP-stimulated cells, they were in several cases represented by a greater number of detectable tryptic peptides, suggesting increased protein abundance and thus increased S-glutathionylation of these proteins. Other identified proteins in both samples likely reflect proteins that are S-glutathionylated by mechanisms independent of ATP stimulation and DUOX1 activation. Targets for ATP-dependent S-glutathionylation include proteins in several functional categories, including cytoskeletal proteins, heat shock proteins, and proteins involved in metabolism or redox regulation (Table 1).

**DUOX1-dependent S-glutathionylation of proteins involved in cell migration**

Previous studies have indicated an important role for extracellular ATP in epithelial wound responses by promoting DUOX1-dependent cell migration [11,12], and cell migration is also associated with increased protein S-glutathionylation [19,20]. We therefore established the importance of DUOX1 in ATP-dependent S-glutathionylation of several selected proteins that have previously been invoked in cell migration, by Western blot analysis of biotin-labeled proteins from BioGEE-loaded stimulated or unstimulated H292 or MTE cells. As expected, ATP-stimulated migration of H292 cells as well as MTE cells depended critically on DUOX1, as this response was suppressed completely in H292-shDUOX1 cells and DUOX1-deficient MTE cells (Fig. 4A and B). Western blot analysis of biotinylated proteins from BioGEE-preloaded H292 cells showed ATP-dependent increases in S-glutathionylation of several proteins with known functions in cytoskeletal regulation or cell migration, namely β-actin, Prx-1, Src, and MKP-1 (Fig. 4C). Moreover, no such increases were observed in H292-shDUOX1 cells that lack DUOX1 (Fig. 4C), indicating that ATP-dependent S-glutathionylation of these proteins is DUOX1-dependent and may represent key events in promoting cell motility and migration. Similar findings were observed in MTE cells, in which ATP stimulation promoted S-glutathionylation of these proteins cells from wild-type mice but not from DUOX1 knockout mice (Fig. 4D). Collectively, these findings confirm that DUOX1 activation contributes to ATP-stimulated epithelial cell migration at several levels, by promoting S-glutathionylation of a number of target proteins involved in cell signaling and cytoskeletal control.

**Discussion**

The present studies build on our recent studies that indicate a role of DUOX1 in epithelial wound responses, and highlight the involvement of S-glutathionylation of a number of proteins as a potential key mechanism in these responses. Wound healing is a complex and essential biological process that involves both immediate actions of conserved damage signals as well as transcription of a variety of genes to further advance the wound response, and a range of studies in diverse organisms and cell systems have identified the common involvement of purinergic molecules (including as ATP), Ca2+, and reactive oxygen species such as H2O2, as mediators in early wound responses [31]. Previous studies with airway epithelial cells have demonstrated a transient rise in extracellular ATP in response to cell activation or injury [12,32], and in turn, the present studies demonstrate rapid and transient activation of DUOX1-dependent H2O2 production in airway epithelial cells in response to extracellular ATP, which is in close agreement with recently reported transient and localized H2O2 generation in injured tail fin of zebrafish larvae [7]. While the overall importance of H2O2 in wound healing responses has been well recognized [31], the direct targets of H2O2-dependent oxidation during wound healing remain to be fully identified. Using both human and mouse epithelial cell model systems, we herein demonstrate the critical importance of DUOX1 in promoting S-glutathionylation of several target proteins in response to cell stimulation with extracellular ATP. Using a global proteomic survey and targeted analysis of candidate proteins, our studies reveal that ATP stimulation and/or DUOX1 activation promotes the S-glutathionylation of a diverse range of proteins involved in either cytoskeletal control, cell metabolism, and redox signaling, and indicating that ATP- and DUOX1-mediated wound responses are not due to a single redox event, but rather involve concerted and integrated redox regulation of a range of proteins.

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**Fig. 3.** Analysis of DUOX1-dependent protein S-glutathionylation using BioGEE. H292-CTL or H292-shDUOX1 cells (A) or MTE cell from either wild-type or DUOX1 knockout mice (B) were preloaded with BioGEE (250 μM; 1 h), stimulated with ATP (100 μM; 15 min), and cell lysates were mixed with non-reducing sample buffer for analysis by SDS-PAGE, and biotin-labeled proteins were detected by blotting with streptavidin-HRP and enhanced chemiluminescence. Representative blots of 2–3 independent experiments are shown.
In spite of the variable extent of ATP-dependent extracellular H$_2$O$_2$ production by H292 cells compared to MTE cells (Fig. 1), the overall extent of ATP-stimulated protein $\text{S}-$glutathionylation was comparable (Figs. 3 and 4). In fact, our findings indicate that the extent of ATP-dependent $\text{S}-$glutathionylation corresponds poorly with extracellular H$_2$O$_2$ production, which merely reflects H$_2$O$_2$ production at the cell surface rather than overall cellular H$_2$O$_2$. Indeed, it is important to note that a substantial fraction DUOX protein is localized to intracellular compartments [14], and that ATP-dependent protein $\text{S}-$glutathionylation may have resulted primarily from intracellularly produced H$_2$O$_2$ or related oxidant species (as indicated by DCF fluorescence; Fig. 1D), rather than paracrine effects of extracellularly generated H$_2$O$_2$. Indeed, ATP-dependent purinergic activation not only results in activation of DUOX1 or other NADPH oxidases, but also evokes cellular responses due to activation of mitochondria-derived reactive oxygen species (e.g. [36]). Intriguingly, our present proteomic analyses indicate that ATP stimulation resulted in markedly increased $\text{S}-$glutathionylation of several mitochondrial proteins, such as pyruvate carboxylase or ATP-citrate synthase (Table 1), which would suggest involvement of mitochondria-derived oxidants. The almost complete inhibition of ATP-dependent oxidant production as well as overall $\text{S}-$glutathionylation in cells lacking DUOX1 would suggest that such mitochondrial oxidant production and $\text{S}-$glutathionylation of mitochondrial proteins may have resulted from initial activation of DUOX1, although this remains to be formally tested in future studies.

Using Western blotting of biotin-labeled proteins in BioGEE-loaded cells, we demonstrated DUOX1-dependent $\text{S}-$glutathionylation of several proteins with known roles in cell signaling and cytoskeletal regulation in response to ATP stimulation. For example, dynamic alterations of the actin cytoskeleton and localized formation of actin filaments at the leading edge are critical for cell migration [36], and a critical cysteine residue in actin, Cys374, was recently identified as a target for reversible $\text{S}-$glutathionylation upon cell stimulation or during cell adhesion [37,38]. Our observation of DUOX1-dependent actin $\text{S}-$glutathionylation in response to ATP stimulation of airway epithelial cells would suggest that such actin $\text{S}-$glutathionylation similarly controls cytoskeletal dynamics and promotes cell migration dynamics. The importance of the dynamics of actin $\text{S}-$glutathionylation and de-glutathionylation in cell migration was recently demonstrated in studies with neutrophils lacking glutaredoxin 1.

**Fig. 4.** DUOX1 activation promotes $\text{S}-$glutathionylation of proteins involved in cell migration. H292-CTL and H292-shDUOX1 cells (A) or MTE cells from wild-type (WT) or DUOX1-deficient (DUOX1-KO) mice (B) were seeded on 8 $\mu$m polycarbonate filters coated with fibronectin and cell migration by haptotaxis was evaluated in the absence or presence of ATP (100 $\mu$M) over 24 h, and quantified and expressed relative to unstimulated H292 cells. Mean S.E. (n=4). *: p < 0.05 compared to corresponding treatment of H292-CTL or WT MTE cells. BioGEE-preloaded H292-CTL or H292-shDUOX1 cells (C) or MTE cells from WT or DUOX1-KO mice (D) were stimulated with ATP and biotinylated proteins were collected using neutravidin beads, and analyzed by SDS-PAGE and Western blotting with antibodies against $\beta$-actin, Prx1, MKP-1, or Src. Corresponding whole cell lysates were evaluated as input controls. Representative blots of 2 independent experiments are shown.
failed to detect some putative S-glutathionylation in response to neutrophil activation that was associated with reduced neutrophil polarization, chemotaxis, adhesion, and phagocytosis [21].

Another target for DUOX1-dependent S-glutathionylation is the MAPK phosphatase MKP-1, which controls MAPK signaling pathways involved in cell motility and migration [12]. Indeed, recent studies in monocytes demonstrated that S-glutathionylation of MKP-1 results in its inactivation and subsequent degradation, thereby promoting monocyte adhesion and migration [22], and suggest that DUOX1-dependent MKP-1 S-glutathionylation might similarly promote epithelial cell migration. Additionally, following recent studies demonstrating a critical role for oxidative activation of Src family kinases in DUOX-dependent cell migration [9,11], our present findings suggest that such oxidative activation of Src may involve S-glutathionylation. Finally, reversible S-glutathionylation is also known to regulate the functions of peroxiredoxins, a family of ubiquitously expressed thiold-specific peroxidase enzymes. Of the various Prx isoforms, Prx1 appears to be particularly sensitive to S-glutathionylation, especially at Cys83, preventing its functional change from low molecular weight oligomers with peroxidase activity to high molecular weight complexes that possess molecular chaperone activity [39,40]. DUOX1-dependent Prx1 S-glutathionylation may be critical in preserving its peroxidase properties to regulate appropriate redox signaling. The involvement of Prx1 in controlling NADPH oxidase-dependent redox signaling and wound responses is supported by recent studies demonstrating transient inactivation of Prx1 by Src-dependent phosphorylation [41]. In addition, Prx1 was also recently demonstrated to interact with MAPK phosphatases such as MKP-1 to control cell signaling pathways [42]. Collectively, the apparent involvement of DUOX1 in S-glutathionylation of these various protein targets, and their known interactions in various cellular processes suggests DUOX1-dependent redox regulation of these processes at various levels as illustrated in Fig. 5. However, the precise cysteine targets for DUOX1-derived H2O2 in these proteins are still unclear, as are the functional consequences of their oxidation, and this will need to be established in future studies.

In summary, the present studies establish an important role for DUOX1 in cellular redox signaling by protein S-glutathionylation, and identify a number of protein targets that are subject to DUOX1-dependent S-glutathionylation in response to ATP, with known functions in cytoskeletal control and cell migration, cell metabolism, and redox regulation. It is important to recognize that our global proteomic survey has some limitations, as it may have failed to detect some putative S-glutathionylation targets, and conversely may have generated some false positives (e.g. resulting from co-purification with biotin-tagged proteins), and their definitive identification as S-glutathionylated proteins would require complementary approaches. The diverse nature of protein targets for DUOX1-dependent S-glutathionylation is consistent with recent studies indicating NOX/DUOX-dependent H2O2 gradients that may act by paracrine signaling e.g. as a chemotactic signal to recruit neutrophils and macrophages to wound sites by more distant redox events [7,16]. However, our present findings to not establish whether identified S-glutathionylated proteins are direct targets for DUOX1-derived H2O2 and we can not rule out the possibility that some of these may have been S-glutathionylated by more indirect mechanisms, e.g. by indirect oxidant production by mitochondria [35] or by potential trans-glutathionylation mechanisms, analogous to previously established thiol-disulfide exchange mechanisms that transmit redox signals [43,44]. Follow-up studies that more directly probe initial thiol oxidation to sulfenic acids (e.g. [45,46]) will be critical to better evaluate such proximal oxidant signals in relation to DUOX1 activation.

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**Appendix A. Supporting information**

Supplementary data associated with this article can be found in the online version at [http://dx.doi.org/10.1016/j.redox.2013.12.030](http://dx.doi.org/10.1016/j.redox.2013.12.030).

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