Research Article

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Hypoxia-induced alterations in the lung ubiquitin proteasome system during pulmonary hypertension pathogenesis

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Abstract

Pulmonary hypertension (PH) is a clinical disorder characterized by sustained increases in pulmonary vascular resistance and pressure that can lead to right ventricular (RV) hypertrophy and ultimately RV failure and death. The molecular pathogenesis of PH remains incompletely defined, and existing treatments are associated with suboptimal outcomes and persistent morbidity and mortality. Reports have suggested a role for the ubiquitin proteasome system (UPS) in PH, but the extent of UPS-mediated non-proteolytic protein alterations during PH pathogenesis has not been previously defined. To further examine UPS alterations, the current study employed C57BL/6J mice exposed to normoxia or hypoxia for 3 weeks. Lung protein ubiquitination was evaluated by mass spectrometry to identify differentially ubiquitinated proteins relative to normoxic controls. Hypoxia stimulated differential ubiquitination of 198 peptides within 131 proteins ($p < 0.05$). These proteins were screened to identify candidates within pathways involved in PH pathogenesis. Some 51.9% of the differentially ubiquitinated proteins were implicated in at least one known pathway contributing to PH pathogenesis, and 13% were involved in three or more PH pathways. Anxa2, App, Jak1, Lmna, Pdcd6ip, Prkch1, and Ywhah were identified as mediators in PH pathways that undergo differential ubiquitination during PH pathogenesis. To our knowledge, this is the first study to report global changes in protein ubiquitination in the lung during PH pathogenesis. These findings suggest signaling nodes that are dynamically regulated by the UPS during PH pathogenesis. Further exploration of these differentially ubiquitinated proteins and related pathways can provide new insights into the role of the UPS in PH pathogenesis.

Keywords

ubiquitin, proteasome, hypoxia

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Pulmonary hypertension (PH) is a clinical disorder characterized by sustained increases in pulmonary arterial pressure and pulmonary vascular resistance caused in large part by remodeling of the pulmonary vasculature. Over time, these derangements lead to right ventricular (RV) hypertrophy, and, if untreated, can ultimately cause RV failure and death. PH can be idiopathic or associated with diverse conditions such as HIV infection, liver disease, congenital heart disease, left-sided heart disease, chronic obstructive pulmonary disease, chronic lung disease with hypoxemia, or hemolytic anemias. PH is associated with significant morbidity, mortality, and high hospitalization rates, indicating a need for better therapeutic approaches as well as earlier detection of PH.[1,2]

During PH pathogenesis, cells in the pulmonary vascular wall become hyperproliferative and resistant to apoptosis, leading to cell proliferation and remodeling of the pulmonary vasculature that causes increases in pulmonary vascular resistance. Previous reports have investigated changes in the

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lung proteome in models of PH;[3–6] however, few studies have examined global changes in protein ubiquitination. The ubiquitin proteasome system (UPS) regulates a variety of fundamental cellular processes including cell proliferation through modulation of protein stability and activity.[7–11] Several reports demonstrate that inhibiting proteasomal activity attenuates aspects of PH in experimental models,[12–16] and others have further defined proteolytic ubiquitination events in models of PH.[4,17–20] However, because nearly half of ubiquitin modifications are estimated to be nonproteolytic[21–23] in function, more global analysis of non-proteolytic ubiquitination as well as linkage use in ubiquitin chain formation is needed to better understand the role of the UPS in PH pathogenesis. Ubiquitin chains can be formed using different lysines on the surface of the initial ubiquitin. These distinct ubiquitin chains may target proteins for diverse outcomes. For example, ubiquitin chains formed by linking sequential ubiquitin moieties via K11 target those proteins for endoplasmic reticulum-associated protein degradation and are involved in cell cycle regulation. On the other hand, K6 chains are involved in DNA damage repair, while K63 chains are involved in cellular signaling and protein trafficking, and K48 chains comprising four or more ubiquitin molecules target proteins for proteasomal degradation.[22] In addition, mono-ubiquitination is involved in DNA damage response, alters the function of proteins, triggers internalization of cell-surface receptors, or primes a protein for poly-ubiquitination.[24] Ubiquitin priming allows faster ubiquitination through chain elongation in response to internal or external cellular stimuli.[25–27]

Limited studies suggest that alterations of the UPS occur in PH, though the specific proteins and pathways involved are poorly defined. For example, proteasome subunit beta 6 (PSB6)[4] and E3 ubiquitin ligases, such as SMAD specific E3 ubiquitin protein ligase 1 (Smurf1)[17] and muscle RING finger 1 (Murf1),[19] are increased in samples from patients and experimental models with PH. The UPS also regulates many other proteins known to play critical roles in PH pathogenesis, such as Hif1α[28-31] and the Rho GTPase signaling cascade.[32–35] Further, reductions in programs of gene transcription regulated by PPARγ, a transcription factor whose levels are reduced in PH, modulate various components of the UPS.[36] Collectively, these observations suggest significant alterations in the UPS during PH pathogenesis that merit additional exploration.

To further investigate UPS alterations in PH, the current study uses a mass spectrometry (MS) screen to identify changes in ubiquitinated peptides in PH. Lung tissues from C57BL/6J mice exposed to normoxia (21% O_2) or hypoxia (10% O_2) for 3 weeks were examined, and in silico analysis was used to identify differentially ubiquitinated peptides that may play a role in PH-associated pathology. Hypoxia-induced changes in ubiquitination were identified in 198 peptides in 131 proteins, 55.7% of which are involved in pathways already associated with PH. These results reveal broad alterations in UPS-mediated protein regulation in the lung during PH pathogenesis. The enrichment of many of these proteins in pathways implicated in regulating PH pathogenesis suggests that improved understanding of these post-translational modifications can contribute to the discovery of new targets for therapeutic intervention.

Methods

Ethical statement

All animal studies were reviewed and approved by the Atlanta VAMC Institutional Animal Care and Use Committee and were performed in accordance with NIH guidelines outlined in the *Guide for the Care and Use of Laboratory Animals*.

Chronic hypoxia mouse model

To assess the effect of hypoxia on proteasome activity and lung protein ubiquitination, male C57BL/6J mice, ages 6–9 weeks, were housed in room air (normoxia, 21% O_2) or in hypoxic chambers (10% O_2) regulated by the ProOx 110 Controller (BioSpherix, Lacona, NY) for 3 weeks. As we and others have previously reported, this regimen induced significant PH, RV hypertrophy, and pulmonary vascular remodeling.[37,38] To confirm this PH phenotype, right ventricular systolic pressure (RVSP) was measured in mice lightly anesthetized with isoflurane. A 0.8 F micro-tip pressure transducer (Transonic, Ithaca, NY) was inserted into the right jugular vein and advanced to the right ventricle. RVSP was monitored for 10 minutes, and data were analyzed using the Powerlab system (ADInstruments, Denver, CO). After RVSP measurement, animals were euthanized by CO_2 exposure and lungs were excised and perfused blood-free with sterile phosphate buffered saline. Right and left lungs or pulmonary artery were transferred to individual 1.5 ml Eppendorf tubes and flash frozen in liquid nitrogen followed by storage in a –80 °C freezer.

Western blotting

Samples were homogenized in lysis buffer (20 mM Tris pH 7.4, 2.5 mM EDTA, 1% Triton X-100, 1% deoxycholic acid, 1% SDS, 100 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4) containing all appropriate inhibitors then centrifuged to remove unbroken tissue. Lysates were boiled then separated at 200 V on NuPAGE 10% Bis-Tris gels (Novex NB0303BOX) using NuPAGE® 2-(N-morpholino) ethanesulfonic acid and sodium dodecyl sulfate (MES-SDS) buffer then transferred onto nitrocellulose membranes at 300 mA. After washing, blots were blocked in appropriate blocking buffer then probed with diluted primary antibody overnight at 4°C. Secondary antibodies (Licor, Lincoln, NE) were prepared at 1:20,000. The following primary antibodies were used: β-actin 1:2000 in 5% non-fat dry milk diluted in

TBS plus 1% Tween (NFDM) (Santa Cruz Biotechnology, sc1616), Fhl1 1:2000 in NFDM (Proteintech, 10991-1-AP), Annexin A2 (R&D Biosystems, MAB3928), NOSTRIN (US Biological, 039064), and Profilin-1 in 5% Bovine Serum Albumin diluted in TBS plus 1% Tween (BSA) (Abcam, ab133529). Immunodetection was performed using a near-infrared fluorescent method (Licor, Lincoln, NE).

Proteasome activity assay

Chymotrypsin-like, trypsin-like, and caspase-like proteasome activities were measured in mouse lung and mouse pulmonary artery after exposure to 3 weeks of normoxia or hypoxia. Lung tissue from a single animal, or pulmonary arteries pooled from four animals within the same treatment group, were prepared in homogenization buffer (25 mM Tris pH 7.5, 100 mM NaCl, 5 mM ATP, 0.2% glycerol). Samples were then centrifuged for 10 min at 5000 rpm at 4 °C, the supernatant was collected, and protein concentrations were determined by bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific, Rockford, IL). To measure proteasomal activity, samples were then incubated for 1 hour with assay buffer [50 mM Tris pH 7.5, 2 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 2 mM ATP] containing the fluorogenic substrate [40 μM Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (AMC), chymotrypsin-like; Boc-Leu-Arg-Arg-AMC, trypsin-like; or Z-Leu-Leu-Glu-AMC, caspase-like; Enzo Life Sciences, Farmingdale, NY]. Samples treated with either 1 μg purified human 20S Proteasome (Enzo Life Sciences) or 40 nM MG132 were used as positive and negative controls, respectively. Proteolytic activities were measured by observing the fluorescence created by release of the AMC fluorescent group with a Wallac Victor 3 fluorimeter (PerkinElmer, Waltham, MA) every 10 minutes over 1 hour (excitation = 390 nm and emission = 460 nm).

Tissue preparation for liquid chromatography–tandem mass spectrometry (LC-MS/MS)

The left lungs from 20 animals, 10 from each condition (normoxic and hypoxic), were used for MS studies. Tissues from two animals were paired to generate enough protein for the MS screen. Tissue from the animal with the highest RVSP in each condition was paired with the animal with the lowest RVSP in the corresponding condition to reduce variability. This process was repeated until tissues from all animals in each condition were paired, generating five samples. Proteasome inhibitors were not included in sample preparation in order to facilitate detection of non-proteolytic ubiquitination events. All tissue homogenization was performed using a Bullet Blender (Next Advance, Troy, NY) according to protocols provided by the manufacturer and as described previously.[39] Briefly, ~300 mg tissue samples were homogenized in 500 μl urea lysis buffer [8 M urea, 100 mM NaHPO4, pH 8.5, including 5 μl (100 × stock) Halt protease and phosphatase inhibitor cocktail (Pierce)]. Each sample was blended twice for 5 min at 4 °C. Protein supernatants were sonicated (Sonic Dismembrator, Fisher Scientific) three times for 5 s at 30% amplitude with 15 s intervals of rest. Samples were then vortexed, protein concentrations were determined by BCA, and samples were frozen in aliquots at –80 °C. Each lung homogenate was analyzed by SDS-PAGE to assess for protein integrity by Coomassie blue staining. Protein homogenates (15 mg) were diluted with 50 mM NH_4HCO_3 to a final concentration of less than 2M urea, then treated with 1 mM DTT, followed by 5 mM iodoacetamide. Protein solutions were digested overnight with 1:100 (w/w) trypsin (Promega, Madison, WI) at 25 °C. Resulting peptides were desalted with a Sep-Pak C18 column (Waters). Acetonitrile was removed by speedvac. The concentrated solution was then frozen and fully lyophilized. The Cell Signalling PTMScan Ubiquitin Remnant Motif kit was used to enrich for ubiquitinated peptides according to the manufacturer's protocol.[40] The resulting peptides were cleaned using an in-house prepared stage-tip then dried by speedvac.

LC-MS/MS analysis

LC-MS/MS analysis was performed as described previously.[39] The dried peptides (2 μg) were re-suspended in peptide loading buffer (0.1% formic acid, 0.03% trifluoroacetic acid, 1% acetonitrile). Peptide mixtures were separated on a self-packed C18 (1.9 μm Dr. Maisch, Ammerbuch-Entringen, Germany) fused silica column (25 cm × 75 μm internal diameter; New Objective, Woburn, MA) by an UltiMate RSLCnano System (Thermo Fisher Scientific, Waltham, MA) and monitored on an Orbitrap Fusion mass spectrometer (ThermoFisher Scientific, San Jose, CA). Elution was performed over a 140-minute gradient at a rate of 300 nl/min with buffer B ranging from 3% to 80% (buffer A: 0.1% formic acid in water, buffer B: 0.1 % formic acid in acetonitrile). The mass spectrometer cycle was programmed to collect at top speed for 3 s. The MS scans (400–1600 m/z range, 200,000 Automatic Gain Control, 50 ms maximum ion time) were collected at a resolution of 120,000 at m/z 200 in profile mode, and the MS/MS spectra (0.7 m/z isolation width, 30% collision energy, 10,000 AGC target, 35 ms maximum ion time) were acquired in the ion trap. Dynamic exclusion was set to exclude previous sequenced precursor ions for 20 s within a 10-ppm window. Precursor ions with + 1, and +8 or higher charge states were excluded from sequencing.

Database search

Spectra were searched using Proteome Discoverer 2.0 against a mouse REFSEQ database (version 62 with 27,216 target sequences). Methionine oxidation (+15.9949 Da), asparagine and glutamine deamidation (+0.9840 Da), lysine ubiquitination (+114.04293) and protein N-terminal acetylation

(+42.0106 Da) were variable modifications (up to three allowed per peptide); cysteine was assigned a fixed carbamidomethyl modification (+57.0215 Da). Only fully tryptic peptides were considered with up to two miscleavages in the database search. A precursor mass tolerance of ±10 ppm and a fragment mass tolerance of 0.6 Da were applied. Spectra matches were filtered by Percolator to a peptide-spectrum match false discovery rate of less than 1%. Peptide-level data were compiled with in-house written software.

Ubiquitin linkage and total ubiquitin analysis

Ubiquitin linkage was identified by searching modified peptides for the following sequences: QIFVK@TLT (K6 linkage), TLTGK@TITL (K11 linkage), TIENVK@A (K27 linkage), AK@IQD (K29 linkage), IQDK@EGIP (K33 linkage), IFAGK@QLE (K48 linkage), and NIQK@EST (K63 linkage). Total ubiquitin was quantified by searching peptides for sequence TITLE. Total ubiquitin peptide areas were then summed for each animal. Each ubiquitin linkage group was normalized to total ubiquitin for each animal.

Data analysis

Differential ubiquitination, volcano plot, and supervised clustering

Differentially ubiquitinated peptides were detected by calculating Student's *t*-test *p*-values ($p < 0.05$ considered significant) and fold-difference $|\log2(\text{hypoxia}/\text{normoxia})| \geq 0.32$ (≥ 1.50-fold-change). When loss of ubiquitination is observed in hypoxia $|\log2(\text{-normoxia}/\text{hypoxia})| \geq 0.32$ (≥ 1.50-fold-change) is reported. Peptide modifications with missing values in four or more of 10 samples were excluded. Volcano plots were plotted with ggplot2 packages in R.[41] The heatmap was plotted with heatmap.2 function in gplots package in R.

Protein ubiquitination in cellular pathways associated with PH pathology

Pathways selected for examination (cell cycle, cell proliferation, cell migration, cell death, calcium signaling, nitric oxide signaling, mitochondria, tissue remodeling and angiogenesis, and vasoconstriction) were based on common associations in the literature. An annotated index of gene ontologies associated with each selected PH pathway was compiled from AmiGO 2 (http://amigo.geneontology.org/amigo). Proteins identified by MS screen were cross-referenced (using gene name and gene symbol) for matches in PH pathway indexes. Gene ontologies within each PH pathway were then grouped by function. Proteins with roles in multiple subcategories were included in both categories.

Gene ontology analysis of proteins not involved in known PH-related pathways

Gene symbols for 63 remaining proteins were compiled then uploaded to Database for Annotation, Visualization and Integrated Discovery (DAVID). The uploaded gene list was analyzed by setting annotations based by species (*Mus musculus*) in annotation databases GOTERM_BP_FAT, GOTERM_CC_FAT and GOTERM_MF_FAT to produce a functional annotation list for biological process, cellular component and molecular function, respectively. Bonferroni corrected *p*-value cutoff (<0.05) and a minimum number of three genes for each GO term were used as filters in pruning the ontologies.

Protein interaction analysis

The chord diagram was generated using R-package GOplot.[42] Protein interactions were assessed using Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) version 10.5 (https://string-db.org). Gene symbols were used as search terms, *Mus musculus* was used as the basis organism. Evidence-based network edges were used which included experimental data and databases. The standard confidence (0.400) was applied. Disconnected nodes were hidden.

Results

Hierarchical cluster analysis of overall changes in ubiquitinated peptides in chronic hypoxia-exposed mouse lungs

To explore the extent of changes in protein ubiquitination in hypoxia-induced PH, MS screening was performed on lungs collected from mice exposed to 3 weeks of normoxia or hypoxia ($n = 5$ with each pooled from two animals). Ubiquitinated proteins were trypsinized to peptides, then treated with urea to remove ubiquitin modifications. Peptides were then precipitated using a proprietary antibody that detects a di-glycine residue left in place of the ubiquitin modification after removal by urea treatment. This method of detection avoids chain specific biases associated with many ubiquitin targeting antibodies. As expected and as previously reported by our group and others,[37] compared with normoxia-exposed animals, this hypoxia exposure regimen caused significant increases in RVSP (Supplementary Fig. S1). Among 2077 peptides screened, hypoxia stimulated alterations in the ubiquitination of 198 peptides in 131 proteins (fold change $\geq \pm 1.50$, $p < 0.05$). As illustrated in Fig. 1a, ubiquitination after hypoxia exposure was significantly increased in 148 peptides (red dots) or decreased in 50 peptides (green dots). The complete list of affected peptides can be found in the supplementary results (Supplementary Table 1). As shown in Fig. 1b, hierarchical clustering was used to visualize patterns of differential ubiquitination between normoxic (left, under white bar) and hypoxic

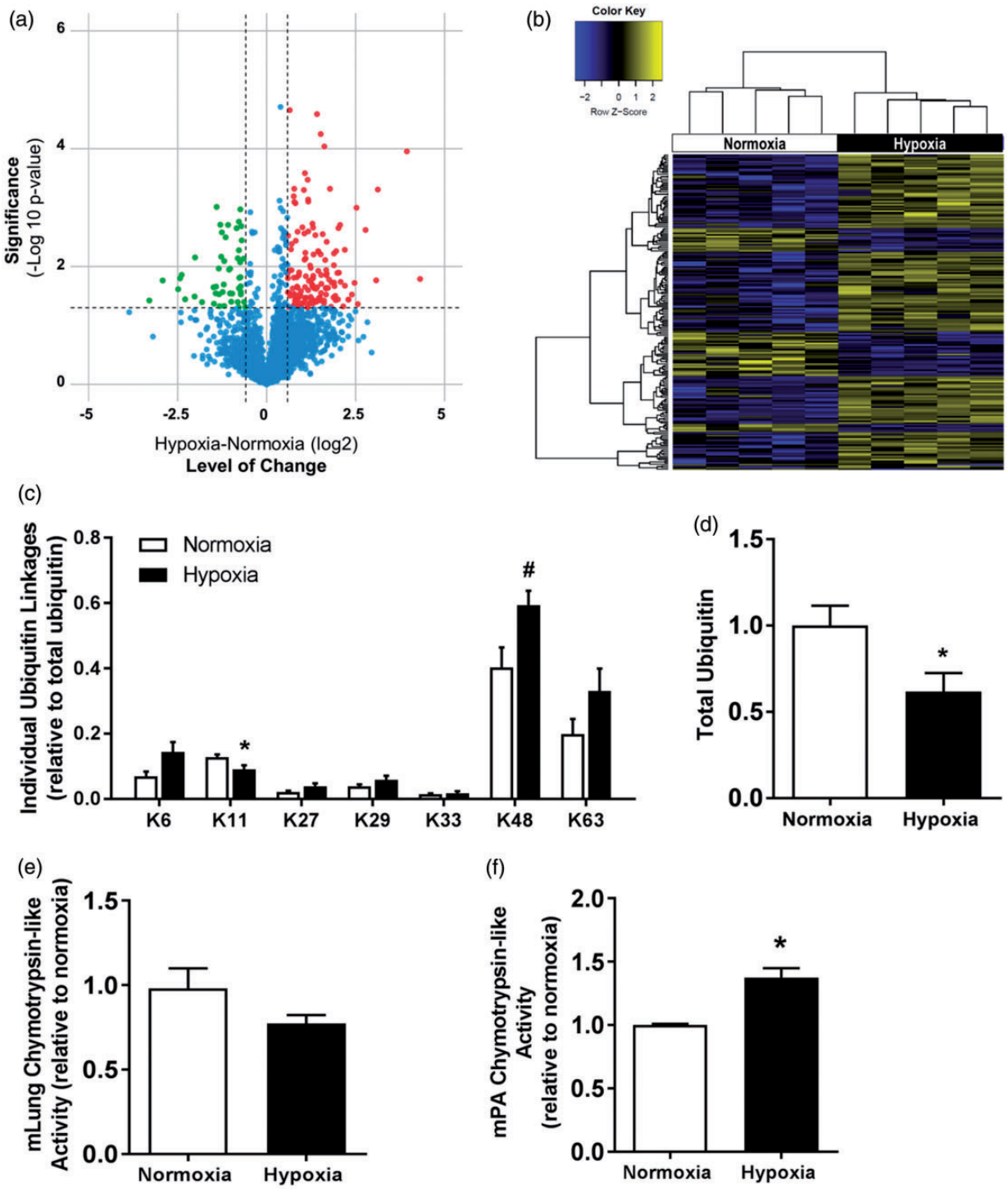

Fig. 1. Chronic hypoxia exposure alters lung protein ubiquitination and proteasome activity. A) Volcano plot comparing total changes in ubiquitination of peptides in the mouse lung after hypoxia exposure. The dashed lines indicate the fold change ($\geq \pm 1.50$) and significance ($p < 0.05$) thresholds. Colored dots represent individual ubiquitinated peptides: peptides represented by blue dots do not meet the significance criteria, peptides represented by green dots demonstrate decreased ubiquitination in hypoxia, and peptides represented by red dots have increased ubiquitination in hypoxia. B) Heat map comparing ubiquitinated peptides in normoxia and hypoxia. Columns represent individual samples; rows represent individual ubiquitinated peptides. C) Ubiquitin chains removed from proteins were analyzed by MS to identify significant changes in ubiquitin chain linkage. D) Total ubiquitin removed from proteins was quantified by summing ubiquitin peptide areas, a measurement of peptide quantity, then normalized to normoxic samples. E and F) Chymotrypsin-like proteasome activity assay was measured in mouse lung (E) and mouse pulmonary artery (F). N=5. *$p < 0.05$ compared to normoxia K11, #$p < 0.05$ compared to normoxia K48. Results are plotted as mean $\pm$ SEM.

(right, under black bar) lung samples. Only modifications with statistically significant changes are included. Each column represents a single sample and the rows represent an individual peptide across all 10 samples. The color intensity represents the deviation from the averaged level of ubiquitination for each peptide in all normoxic and hypoxic samples. Yellow indicates higher than average and blue represents lower than average levels of ubiquitination among samples within this dataset.

Ubiquitin linkages associated with hypoxia exposure

Ubiquitin moieties can be added to an initial ubiquitin tag on the surface of a target protein to form chains. Addition of chains to different regions of the ubiquitin molecule creates different chain types with topologically unique structures and functionally distinct outcomes. Ubiquitin molecules with a di-glycine tag are indicative of ubiquitin chains. Analysis of ubiquitin linkages removed from proteins during the sample preparation process revealed hypoxia-induced reductions in K11-linked ubiquitin chains and increased levels of K48-linked ubiquitin chains (Fig. 1c). K11-linked ubiquitin chains are most commonly associated with cell cycle regulation, cell division, and endoplasmic reticulum-associated protein degradation, while K48-linked ubiquitin chains represent canonical ubiquitin modification mediating proteasomal degradation.[22,24] Hypoxia also reduced total ubiquitin involved in protein modifications by more than 30% (Fig. 1d).

Proteasome activity in mouse lung and pulmonary artery in chronic hypoxia

Because increases in K48 ubiquitination are most commonly associated with proteasome-mediated protein degradation, proteasome activity in mouse lungs was examined. Hypoxia exposure had no significant effect on trypsin-like (data not shown), caspase-like (data not shown), or chymotrypsin-like (Fig. 1e) proteasomal activity in lung homogenates. In isolated pulmonary artery tissues, on the other hand, hypoxia increased chymotrypsin-like activity (Fig. 1f) but did not affect trypsin-like or caspase-like activity (data not shown).

Protein ubiquitination in cellular pathways associated with PH pathology

In order to further examine the role of protein ubiquitination in PH pathogenesis, a focused gene ontology survey was performed across ontologies related to broad pathways commonly associated with PH. PH pathways selected for examination were cell cycle, cell proliferation, cell migration, cell death, calcium signaling, nitric oxide signaling, mitochondria, tissue remodeling and angiogenesis, and vasoconstriction, as dysregulation in these pathways has been implicated in PH. AmiGO 2 was used to compile an annotated index of associated gene ontologies for each PH pathway. Relevant cellular processes with the highest percentage of ubiquitinated peptides identified by MS screen (Fig. 1a and a) included calcium signaling (21.2%), cell migration (19.2%), cell cycle (18.2%), cell proliferation (17.7%), and cell death (15.2%) (Fig. 2). Combined, 102 uniquely modified peptides in 68 proteins participate in PH pathways, comprising 51.5% of the identified peptide modifications and 51.9% of the identified proteins (Table 1).

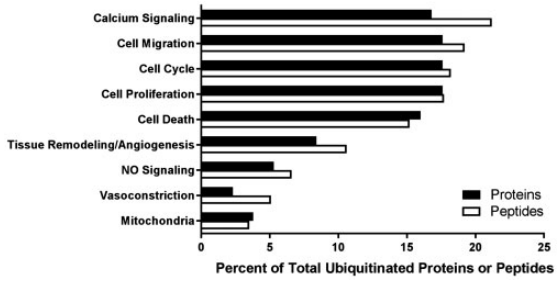

Fig. 2. Ubiquitin modification in pathways commonly associated with PH. AmiGO 2 was used to compile an annotated index of associated gene ontologies for PH associated pathways. Each bar represents the percent of total peptides (filled bars) or proteins (open bars) with significant ubiquitin modifications found within each of the indicated gene ontologies.

Table 1. Summary of total ubiquitin modifications identified in PH-related pathways.

	Proteins	Peptides
Total	131	198
PH Pathways	68	102
% of Total	51.9%	51.5%

Categorizing PH pathways by function using gene ontologies

Gene ontologies within each PH pathway were further classified by function to identify individual mechanisms within the broader PH pathway that are affected by protein ubiquitination (Fig. 3). Proteins within the ontologies for each pathway were excluded from consideration if not associated with a tissue type or organ directly associated with PH. For example, ontologies relating to neural cells, hair follicles, osteoblasts and viruses were excluded. Complete data are reported in Supplementary Tables 2–6. Proteins within the cell cycle pathway were grouped into subcategories including exocytosis, meiosis, metaphase, telophase, general mitotic pathways, prophase, anaphase, and cell cycle checkpoints (Fig. 3a). Some 56.5% of proteins within the cell cycle PH pathway are involved in cell cycle checkpoints, including DNA damage checkpoints and 87% are involved in mitosis (cell cycle checkpoint, anaphase, prophase, metaphase, telophase, and general mitotic pathways).

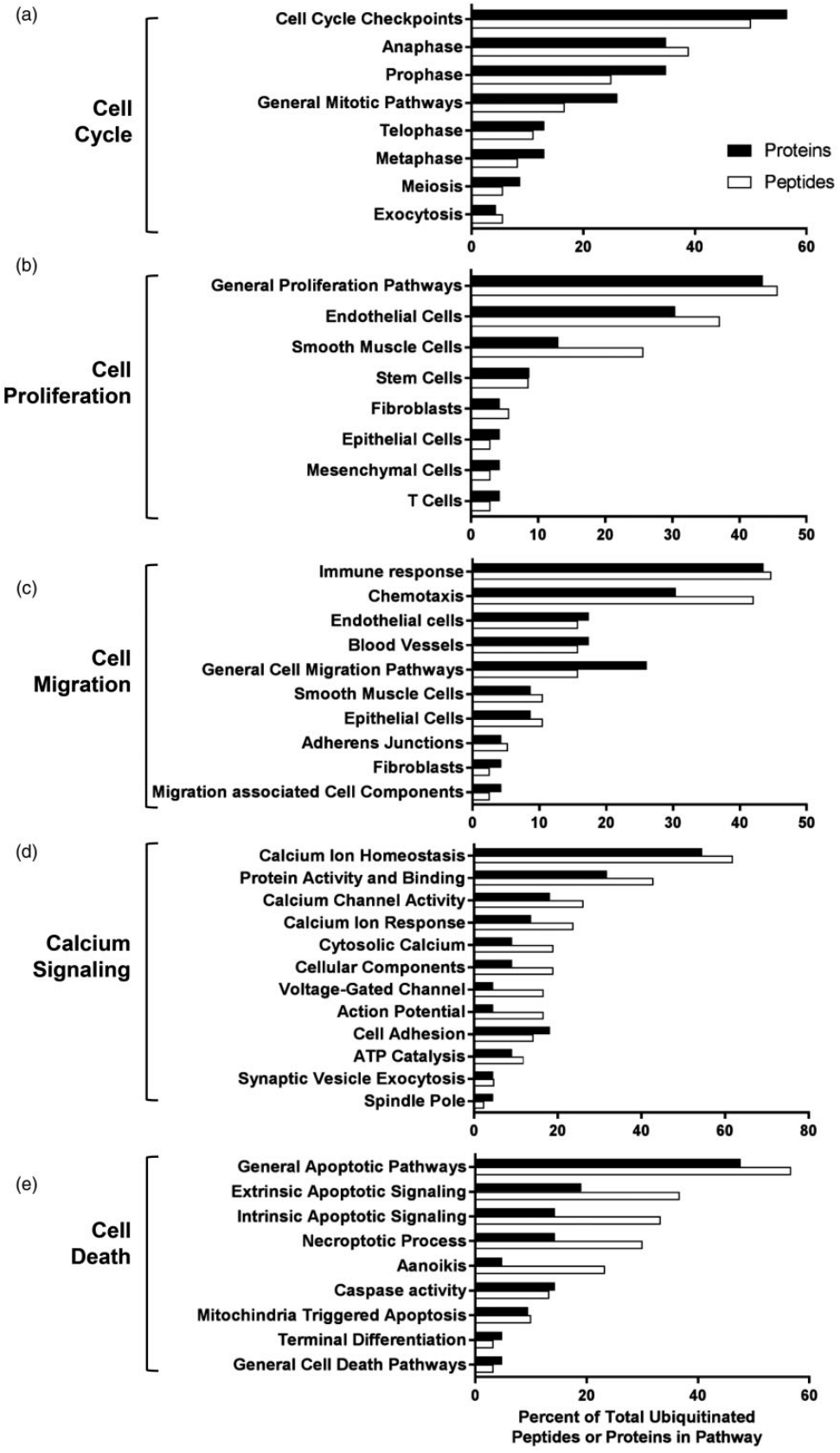

Fig. 3. Protein ubiquitination in PH pathway functional groups. Gene ontologies within each PH pathway selected were grouped by function. Peptides (open bars) or proteins (filled bars) within each functional group are represented as percent of the proteins/peptides in that PH pathway. Functional groups within A) Cell Cycle, B) Cell Proliferation, C) Cell Migration, D) Calcium Signaling, and E) Cell Death are presented.

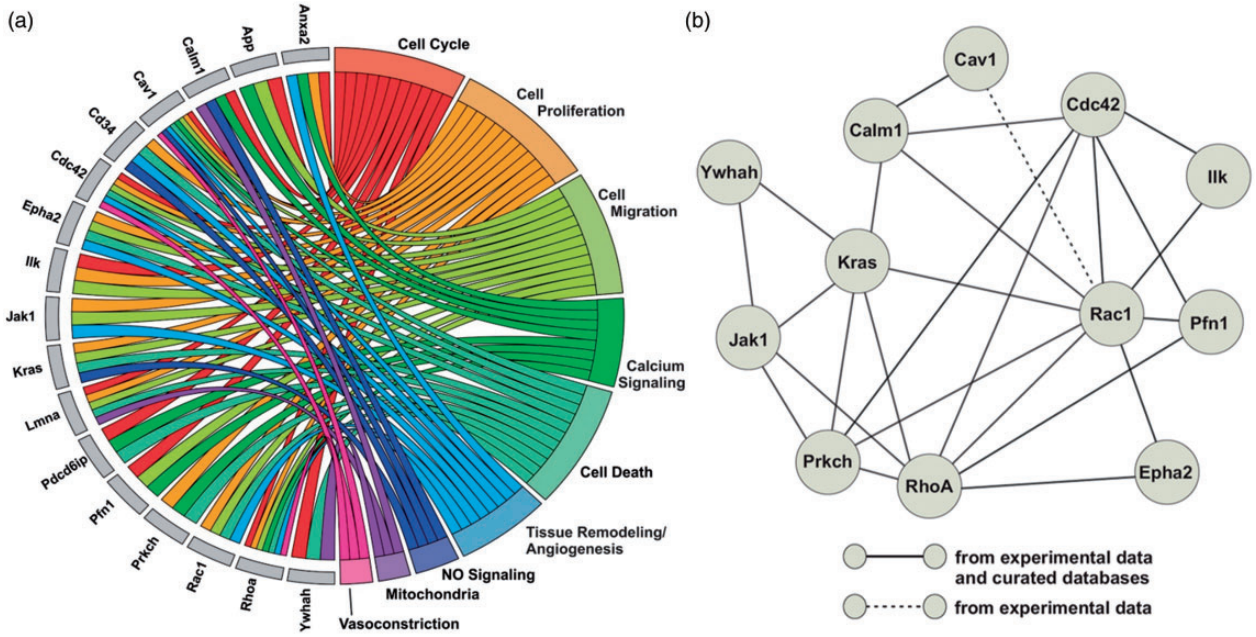

Fig. 4. Ubiquitinated proteins associated with multiple PH pathways. A) The chord diagram demonstrates the overlapping roles of proteins with hypoxia-induced alterations in ubiquitination that function in 3 or more PH pathways. Each protein on the left side of the circle represents a protein that functions in 3 or more PH pathways. PH pathways are on the right side of the circle. B) Graphical representation of protein-interaction network performed on proteins that function in 3 or more PH pathways generated using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins). Solid lines between protein nodes (ovals) indicate protein-protein interactions identified from both experimental data and curated databases. Dashed lines between protein nodes represent protein-protein interactions identified through experimental data only. Anxa2 = Annexin A2, App = Amyloid-beta A4 protein, Calm1 = Calmodulin, Cav1 = Caveolin-1, Cd34 = Hematopoietic progenitor cell antigen CD34 isoform 2 precursor, Cdc42 = Cell division control protein 42 homolog isoform 1 precursor, Epha2 = Ephrin type A receptor 2 precursor, Ilk = Integrin linked protein kinase, Jak1 = Tyrosine-protein kinase JAK1, Kras = GTPase KRas, Lmna = Prelamin-A/C, Pdcd6ip = Programmed cell death 6-interacting protein, Pfn1 = Profilin-1, Prkch = Protein kinase C eta type, Rac1 = Ras-related C3 botulinum toxin substrate 1, Rhoa = Transforming protein RhoA, and Ywhah = 14-3-3 protein eta.

The cell proliferation pathway included proteins involved in proliferation of T cells, mesenchymal cells, epithelial cells, fibroblasts, stem cells, smooth muscle cells, endothelial cells, and general cell proliferation pathways (Fig. 3b). The most well-represented cell proliferation pathway subgroup was general cell proliferation pathways, including 43.5% of all proteins in the cell proliferation pathway. Subgroups identified within the cell migration pathway were migration associated cell components, migration associated with blood vessels, general cell migration pathways, immune response, chemotaxis, adherens junctions, migration of fibroblasts, epithelial cells, smooth muscle cells, and endothelial cells (Fig. 3c). Migration associated with immune response included 43.5% of all proteins in the cell migration pathway. The calcium signaling pathway comprised proteins involved in cytosolic calcium, voltage-gated channels, spindle pole, calcium ion homeostasis, calcium ion response, action potentials, protein activity and binding, cell adhesion, synaptic vesicle exocytosis, ATP catalysis, calcium channel activity, cellular components; with calcium ion homeostasis having the highest representation with 54.5% of proteins in the calcium signaling pathway (Fig. 3d). Lastly, proteins within the cell death PH pathway were categorized as involved in terminal differentiation, mitochondria-triggered apoptosis, intrinsic apoptotic signaling, extrinsic apoptotic signaling, caspase activity, general apoptotic pathways, general cell death pathways, necroptotic process, and anoikis (Fig. 3e). General apoptotic pathways represented the largest sub-category with 47.6% of proteins in the cell death pathway.

Ubiquitinated proteins associated with multiple PH-related pathways

Because individual proteins may have functional roles in multiple pathways, the relationship between proteins with significant alterations in ubiquitination and PH-associated pathways was mapped with a chord diagram. The chord diagram (Fig. 4a) was generated using GOPlot package for R. Protein symbols are located on the left side of the circle, chords connect protein symbols to associated pathways on the right side of the circle. Roughly 13% of all identified proteins play a role in three or more PH pathways (Table 2). Within the 17 proteins that interact in three or more pathways, there are significantly more interactions (PPI enrichment p-value$=1.14 \times 10^{-9}$) than expected for a random set of proteins (Fig. 4b). Since these proteins regulate several pathways associated with PH and changes in

Table 2. Proteins with roles in three or more PH pathways.

8/9 Pathways	Modifications Identified
caveolin 1 isoform 1 (Cav1)	(K26) (K26; K30) (K26; $M32^{OX}$) (K30) (K30; K39) (K30; $M32^{OX}$) (K30; $M32^{OX}$; K39)
7/9 Pathways	
transforming protein RhoA (RhoA)	($C107^{CM}$; K118) ($C107^{CM}$; K119)
6/9 Pathways	
cell division control protein 42 homolog isoform 1 precursor (Cdc42)	(K131) (K133)
5/9 Pathways	
prelamin A/C isoform A precursor (Lmna)	(K135)
4/9 Pathways	
annexin A2 (Anxa2)	(K115) (K80)
calmodulin (Calm1)	(K78)
ephrin type A receptor 2 precursor (Epha2)	(K579)
GTPase KRas (Kras)	(K128)
hematopoietic progenitor cell antigen CD34 isoform 2 precursor (Cd34)	($N330^{DIA}$; K349)
ras-related C3 botulinum toxin substrate 1 precursor (Rac1)	(K147)
3/9 Pathways	
14-3-3 protein eta (Ywhah)	(K50)
integrin-linked protein kinase (Ilk)	(K154; $M155^{OX}$)
profilin-1 (Pfn1)	(K126) (K54)
programmed cell death 6-interacting protein isoform 1 (Pdcd6ip)	(K506)
protein kinase C eta type (Prkch)	(K36)
tyrosine protein kinase JAK1 (Jak1)	(K227)
amyloid beta A4 protein isoform 1 precursor (App)	(K751; $Q754^{DIA}$)
2/9 Pathways	
activated CDC42 kinase 1 isoform 1 (Tnk2)	(K131)
tyrosine protein kinase HCK isoform p59Hck (Hck)	(K38)
apolipoprotein A I preproprotein (Apoa1)	(K205)
claudin-18 isoform A2.1 (Cldn18)	(K229)
hemoglobin subunit beta-1 (Hbb-b1)	(K121)
hepatocyte growth factor regulated tyrosine kinase substrate isoform 1 (Hgs)	($C75^{CM}$; K86)
neuronal membrane glycoprotein M6-a isoform 1 (Gpm6a)	(K257)
peptidyl-prolyl cis-trans isomerase D (Ppid)	($C275^{CM}$; $C282^{CM}$; K283)
peroxiredoxin-6 (Prdx6)	(K97) ($M188^{OX}$; K199; $C201^{CM}$)
radixin isoform a (Rdx)	(K209) (K211) (K212) (K335)
serine/threonine-protein phosphatase 2A catalytic subunit beta isoform (Ppp2cb)	($C20^{CM}$; K21) (K41)
SUN domain-containing protein 1 isoform 1 (Sun1)	(K160)
SUN domain-containing protein 2 isoform 1 (Sun2)	(K124; $N126^{DIA}$)
ubiquitin-60S ribosomal protein L40 (Uba53)	(K11)
vimentin (Vim)	(K188) (K188; $M193^{OX}$) (K282)

K: Ubiquitinated Lysine Residue; M^{OX}: Oxidized Methionine Residue; N^{DIA}: Asparagine Residue Deamidation; Q^{DIA}: Glutamine Residue Deamination; C^{CM}: Cystine Residue Carbamidomethyl Modification

levels of some of these proteins are already implicated in PH (Table 3), it is likely that ubiquitination is a redundant mechanism regulating these critical interactions. However, a literature search revealed that Annexin A2 (Anxa2), Amyloid beta A4 protein (App), Tyrosine protein kinase JAK1 (Jak1), Prelamin A/C (Lmna), Programmed cell death 6-interacting protein (Pdcd6ip), Protein kinase C eta type (Prkch), and 14-3-3 protein eta (Ywhah) have not previously been associated with PH, suggesting that ubiquitination may be a novel regulatory mechanism for these proteins. The chord diagram presents potential regulatory mechanisms by which these proteins may participate in PH pathogenesis and suggests potential targets for future PH research.

Table 3. Literature mining for PH association with top proteins.

Protein	PH Association	Reference
Calmodulin 1	• ↓ Calm1 association with eNOS in intrapulmonary arteries from hypoxic prenatal sheep	Liu J, et al. 2009
	• ↓ Calm1 association with eNOS in pulmonary arteries from hypoxic rats	Murata T, et al. 2002
Caveolin-1	• progressive ↓ in Cav1 in monocrotaline (MCT)-induced rat PH model	Huang J, et al. 2012
	• ↑ Cav1 expression in pulmonary artery SMC associated with neointima formation in MCT rat and PH patients	Huang J, et al. 2015
	• Cav1 mutations associated with familial and idiopathic PAH	Austin ED, et al. 2012
	• predicted Cav1 loss-of-function mutations associated with familial PAH	Desai AA. 2012
	• ↑ Cav1 association with eNOS in pulmonary arteries from hypoxic rats	Murata T, et al. 2002
	• ↑ Cav1 association with eNOS in intrapulmonary arteries from hypoxic prenatal sheep	Liu J, et al. 2009
Profilin-1	• ↑ Pfn1 expression in MCT rat model of PAH	Dai YP, et al. 2006
Hematopoietic progenitor cell antigen CD34	• ↑ Cd34 expression in PH patient pulmonary artery EC	Müller AM, et al. 2002
	• ↑ Cd34 expression association with ireversible lesions in PAH patient pulmonary artery	Huang H, et al. 2011
Ephrin type-A receptor 2	• ↓ Epha2 expression assiciated with worse outcomes in hypoxic-sugen PAH mouse model	Rhodes CJ, et al. 2015
Integrin-linked protein kinase	• ↑ Ilk in distal PASMC from PAH patients and pulmonary artery from rat sugen-hypoxia PAH model	Kudryashova TV, et al. 2016
Cell division control protein 42 homolog isoform 1	• ↑ Cdc42 activity in hypoxic pulmonary artery myocytes	Fediuk J, et al. 2014
	• ↑ Cdc42 expression in lungs of chronic hypoxia mouse	Pi L, et al. 2018
	• ↓ Cdc42 in pericytes purified from the PAH patient lung	Yuan K, et al. 2014
GTPase KRas	• ↑ Kras activity associated with PAH development secondary to lung cancer	Pullamsetti SS, et al. 2017
Ras-related C3 botulinum toxin substrate 1	• ↑ Rac1 expression in arteries from IPAH patients	Laumanns IP, et al. 2009
	• ↑ Rac1 activity in lungs and PASMC from chronic hypoxia PH mouse model	Yu M, et al. 2012
	• ↑ Rac1 activity in lung from BMP2 knock down mouse model of PAH	Johnson JA, et al. 2012
	• ↓ Rac1 activity in PAEC from chronic hypoxia piglets	Wojciak-Stothard B, et al. 2006
Transforming protein RhoA	• ↑ RhoA activity in PAEC from chronic hypoxia piglets	Wojciak-Stothard B, et al. 2006
	• ↑ RhoA expression in arteries from IPAH patients	Laumanns IP, et al. 2009
	• ↑ RhoA activity in lung from BMP2 knock down mouse model of PAH	Yasuda T, et al. 2011
	• ↑ RhoA activity in platelets from PH patients	Yaoita N, et al. 2014

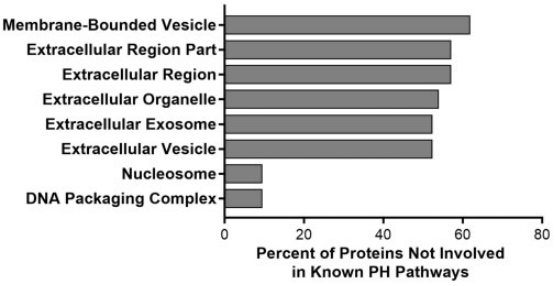

Fig. 5. Gene ontology enrichment in proteins not involved in PH associated pathways. DAVID (Database for Annotation, Visualization and Integrated Discovery) was used to generate a list of gene ontologies. Each bar represents the percent of analyzed proteins associated within the indicated pathway.

Gene ontology analysis of proteins not involved in known PH-related pathways

In addition, we examined the remaining 63 proteins with significant hypoxia-associated changes in ubiquitination that were not involved in pathways associated with PH. The complete list of peptides and modifications can be found in Supplementary Table 7. To identify major pathways represented within these proteins we performed a gene ontology analysis using DAVID. Seven cellular components were enriched in this subset of proteins, including ontologies related to DNA packaging, cellular components found in the extracellular region, vesicles and exosomes (Fig. 5). Enrichment of these pathways suggests that ubiquitination may also modulate hypoxia-related extracellular signaling and may contribute to hypoxia-associated transcriptional

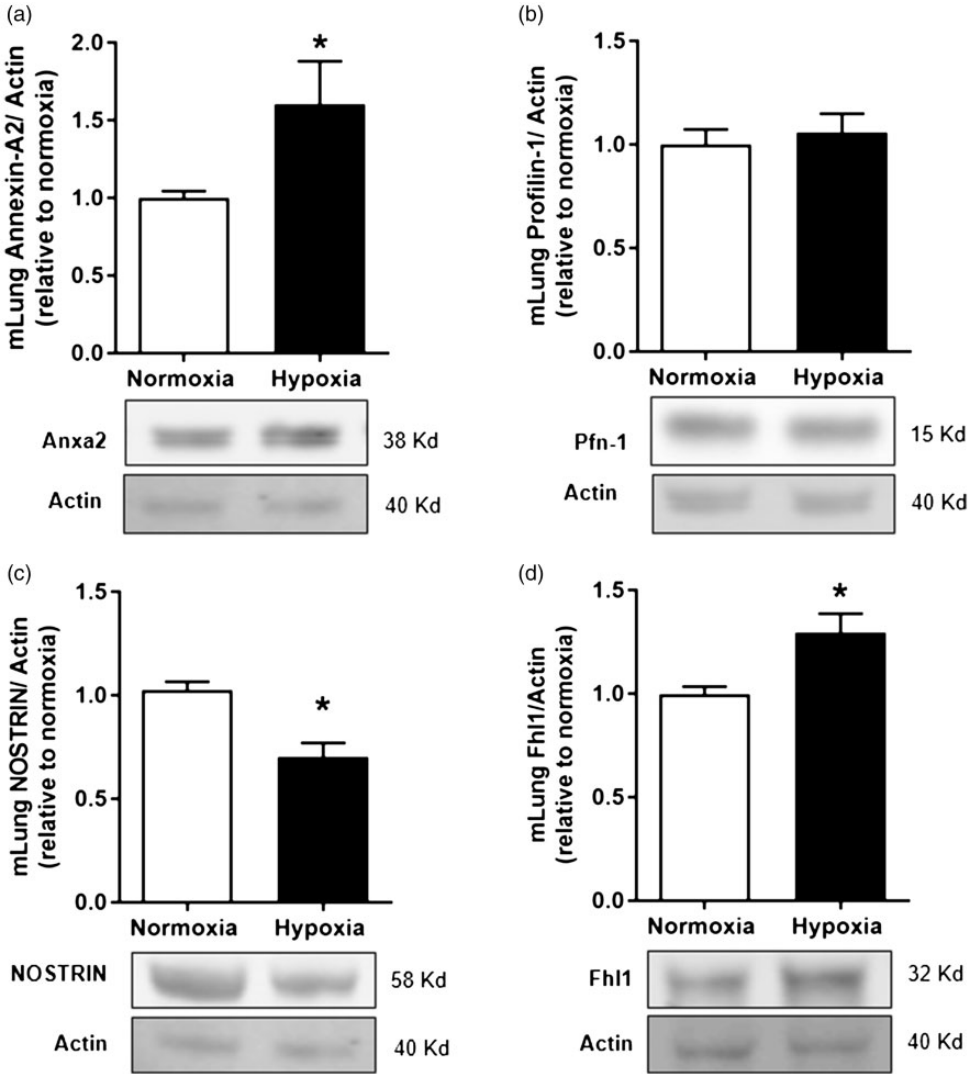

Fig. 6. Validation of selected proteins by western blotting. A) Annexin A2 (n = 13), B) Profilin-1 (n = 11), C) NOSTRIN (n = 12,13), and D) Fhl1 (n = 11,10) protein levels in mouse lung (mLung) were normalized to β-actin ($p<0.05$; * compared to Normoxia). Error bars = SEM. Anxa2 = Annexin A2, Pfn-1 = Profilin-1, NOSTRIN = Nitric Oxide Synthase TRaffic INducer, and Fhl1 = Four and a half LIM domain 1.

changes by altering DNA packaging and chromatin availability to transcriptional machinery.

Validation of selected proteins by western blotting

Finally, four proteins (Annexin A2, Profilin-1, NOSTRIN, and Fhl1) with little or no prior implication in Group 3 PH were selected for initial validation by western blotting. We observed significant increases in ubiquitination of Annexin A2 at K80 (1.5-fold change Hypoxic/Normoxic [FC H/N]) and K115 (2.1 FC H/N) (Supplementary Table 1). Annexin-A2 is a calcium binding protein that accumulates at the cell membrane and is associated with actin assembly, epithelial cell polarization,[43] endo- and exocytosis, and mRNA transport.[44] By western blotting we observed a significant increase in Annexin A2 protein levels (Fig. 6a), suggesting that Annexin A2 ubiquitination observed in hypoxia does not target the protein for degradation. Interestingly, Annexin ubiquitination has previously been associated with its subcellular localization as well as its role in actin-binding[45] and mRNA transport.[44]

The ubiquitination of another actin-related protein, Profilin-1, was also altered by hypoxia. Profilin-1 is a small ubiquitously expressed actin-binding protein[46] that promotes cell protrusion, motility, invasion,[47] and is a potent regulator of actin dynamics that promotes incorporation of globular actin into filamentous actin.[48–50] MS revealed a decrease in profilin-1 ubiquitination in hypoxia at K54 (1.9 FC Normoxic/Hypoxic) and K126 (5.2 FC N/H) (Supplementary Table 1) but no change in profilin-1 protein levels (Fig. 6b). Interestingly, lysine mutations in profilin-1 are known to either enhance or inhibit profilin-1 interaction with actin.[51,52] Profilin-1 mutants that cause familial amyotrophic lateral sclerosis form insoluble ubiquitinated aggregates and are associated with decreased actin binding activity and a reduced filamentous/globular actin ratio.[53]

MS also revealed increased ubiquitination of Nitric Oxide Synthase TRaffic INducer (NOSTRIN) at K190 (1.6 FC H/N) and K417 (1.8 FC H/N) in hypoxia (Supplementary Table 1). NOSTRIN interacts with endothelial NO synthase as well as Caveolin-1 to regulate eNOS trafficking and targeting.[54–56] Western blotting revealed a significant decrease in NOSTRIN protein in lungs from hypoxia-exposed mice (Fig. 6c). Decreased NOSTRIN protein may occur through ubiquitin-mediated proteolytic degradation, though ubiquitination may also alter protein function or subcellular localization. Given that we observed significant changes in ubiquitination at multiple NOSTRIN sites, the impact of the ubiquitination events on NOSTRIN function will required additional studies.

Finally, MS revealed that hypoxia altered the ubiquitination of Four and a half LIM domain 1 (Fhl1). Fhl1 is involved in muscle development[57] and plays an important role in gene transcription and cell proliferation, differentiation, apoptosis and cytoskeletal remodeling.[58] There is previous evidence that Fhl1 is important in vascular remodeling in PH,[57] though we were unable to find any reports of Fhl1 ubiquitination. We observed increased ubiquitination of Fhl1 at K295 (3.1 FC H/N) and decreased ubiquitination at K173 (2.1 FC N/H). Similar to other studies,[57] we observed increased protein levels in hypoxia-exposed mouse lungs (Fig. 6d). Given that we observed a more complex ubiquitination pattern for Fhl1, ubiquitination may be involved in modulating Fhl1 localization or protein–protein interactions.

Discussion

PH, defined as mean pulmonary arterial pressure >25 mm Hg, is an important clinical problem with complex pathogenesis.[59,60] Effective therapies for this condition are lacking, and our understanding of the basic mechanisms of this disorder is incomplete. The rationale for this investigation was based in part on the recognition that PH pathogenesis involves alterations in multiple cellular pathways. In this study we sought to advance the field by further exploring interactions between PH and the UPS. For example, the UPS plays a critical role in aberrant cell proliferation,[61–64] a derangement central to our current understanding of mechanisms of pulmonary vascular remodeling in PH pathogenesis. Further evidence for the role of the UPS in PH pathogenesis stems from a growing number of studies showing that proteasomal inhibitors attenuate experimental PH.[12–16] In addition, ubiquitination has been explored in the context of individual proteins of importance in PH such as Hif1α,[28,31] Rho GTPases,[32–34] and PPARγ,[36] but the overall extent of ubiquitin changes in the lung during PH pathogenesis has not been previously reported. Identification of specific proteins that contribute to PH pathogenesis, especially those that affect multiple pathways in PH pathogenesis, may inform new and more specific and effective therapies for PH.

To further explore the role of the UPS in PH, this study evaluated the effects of hypoxia on global non-proteolytic protein ubiquitination using a MS screen of lungs collected from mice following exposure to normoxia (21% O_2) or hypoxia (10% O_2) for 3 weeks, a time at which hypoxia-exposed animals demonstrate PH with significant increases in RVSP. Our results indicate that hypoxia, the stimulus for PH in these studies, alters the ubiquitination of many proteins that participate in pathways implicated in PH pathogenesis. Using MS screening, 198 peptides were identified with significant changes in ubiquitination, more than 55% of which are involved in pathways known to contribute to PH pathogenesis. These findings highlight the potential importance of ubiquitination as a mediating factor in PH. The PTMScan Ubiquitin Remnant Motif kit (Cell Signaling Technologies) employed in these studies, by detecting a di-glycine residue left after removing the ubiquitin chain, avoids binding biases associated with antibody-based ubiquitin identification strategies.[65] This approach permits definition of protein ubiquitination regardless of ubiquitin

chain linkage or length while also permitting the examination of changes in ubiquitin chain linkage. This approach also permits identification of proteins with post-translational modifications that may alter protein activity rather than protein levels. Such changes to these proteins may not appear when examining changes in protein levels by western blot or traditional MS. This method identified significant changes in the ubiquitination of several proteins that to our knowledge have not been previously examined in PH, including Anxa2, App, Jak1, Lmna, Pdcd6ip, Prkch, and Ywhah. While existing evidence indicates that Anxa2,[66] Jak1,[67] Pdcd6ip,[68] and Prkch[69] are sensitive to changes in oxygen levels, Lmna and Ywhah have not previously been examined in hypoxic conditions. Because proteins implicated in multiple PH-related pathways demonstrate differential protein ubiquitination, these findings suggest that ubiquitin dysregulation may play a larger role in PH than previously recognized. Targeting proteins with functions in multiple PH-related pathways represents an attractive potential strategy for future therapeutic intervention to more effectively address the complex and overlapping pathobiological mechanisms contributing to PH pathogenesis.

As illustrated in Fig. 1c, hypoxia increased proteins with K48-linked chains, the most studied ubiquitin chain commonly associated with proteasome degradation of modified proteins.[22] The increase in K48 ubiquitin chain linkages may indicate an increase in proteins targeted for the proteasome either through K48-linked chains or through the use of mixed linkage multi-ubiquitin chains that may also include K48-linked ubiquitin. Although hypoxia-induced increases in K48 linked ubiquitinated proteins could also indicate an accumulation of proteasome-targeted proteins, hypoxia-induced reductions in proteasomal activity were not observed in either whole lung homogenates or isolated pulmonary artery tissue (Fig. 1e, f). In fact, pulmonary artery proteasome activity was increased by hypoxia, consistent with findings in pulmonary artery smooth muscle cells exposed to hypoxia in vitro and in pulmonary arteries from hypoxia-exposed rats.[4] Increased hypoxia-induced pulmonary arterial proteasomal activity is also consistent with evidence that proteasome inhibition attenuates PH in several models.[4,12,14,15,70] K48-linked ubiquitin chains can also target proteins to the lysosome,[71] participate in organelle biogenesis after mitosis,[72] and recruit proteins to the site of DNA damage.[73] The identification of specific proteins with enhanced hypoxia-induced K48-linked ubiquitination will permit more targeted future studies to explore how these post-translational modifications contribute to proliferative pathways.

Our results also revealed hypoxia-induced reductions in K11-linked ubiquitin chains. These results may indicate a reduction in the activity of the E3 ligase mainly associated with production of K11-linked ubiquitin chains, Anaphase-Promoting Complex.[74] Conversely, loss of K11-linked ubiquitin may also indicate an increase in turnover of proteins targeted for proteasomal degradation by K11 ubiquitin chains. Thus, while the current studies do not fully resolve the mechanisms for these hypoxia-induced alterations in lung protein ubiquitination and linkage, they provide the most detailed examination to date of UPS-mediated alterations in lung proteins during PH pathogenesis that can inform future mechanistic studies.

While the UPS is traditionally considered to mediate protein degradation, the large number of lung proteins undergoing alterations in ubiquitination during exposure to hypoxia (Fig. 1a) combined with the overall reduction in ubiquitin used in protein modification (Fig. 1d) is consistent with hypoxia-induced increases in mono-ubiquitination or short ubiquitin chains. These findings suggest that post-translational modifications more commonly associated with altered protein function than degradation may be particularly important. These findings may also indicate increases in ubiquitin priming, in which a protein tagged with a single ubiquitin or a short ubiquitin chain "primes" it for faster ubiquitination through chain elongation in response to internal or external cellular stimuli.[25–27] Since ubiquitin chain elongation often requires the activity of multiple E3 ligases,[26] ubiquitin priming may be a mechanism of rapidly altering protein stability or activity in response to stimuli.

To explore the potential roles of proteins with altered ubiquitination, we used gene ontology analysis to group proteins into broad pathways associated with PH (Fig. 2). As expected, in hypoxia-exposed mouse lung we observed significant changes in ubiquitin modification of proteins involved in vasoconstriction, NO signaling, tissue remodeling/angiogenesis, mitochondria, cell death, cell proliferation, cell cycle, cell migration, or calcium signaling pathways. This analysis suggests that ubiquitination may play a larger role in regulating pathways commonly associated with cell proliferation. Further classification within each pathway (Fig. 3a–e) reveals that the majority (87%) of identified proteins in the cell cycle pathway are involved in some aspect of mitosis. It is well known that protein ubiquitination is a major mechanism regulating mitotic pathways.[75] This may also suggest dysregulation of the Anaphase-Promoting Complex, the ubiquitin ligase that regulates mitosis.[76] Within the cell cycle pathway (Fig. 3a), cell cycle checkpoints are most heavily affected by hypoxia-induced changes in ubiquitination. Ubiquitination of checkpoint proteins is an important mechanism of cell cycle dysregulation in cancer.[7,77-80] Therefore, our data support that existing checkpoint targeted cancer therapies may have some application in PH.[7,77,81] Interestingly, proliferation of endothelial cells and smooth muscle cells, two of the cell types in the pulmonary vascular wall that undergo proliferation during PH pathogenesis, are the second and third largest sub-classes (Fig. 3b) in the cell proliferation group enriched with ubiquitin modifications. These data highlight the need to continue examining protein ubiquitination as an important mediator in PH. Conversely, in proteins not associated with previously known PH pathways

(Fig. 5) we observed significant changes in ubiquitination of proteins involved in extracellular signaling, including exosomes and vesicles, as well as changes in proteins associated with DNA packaging and chromatin. These pathways may provide interesting new directions for PH research.

Our study has several important limitations that merit additional consideration. Because the design of our studies did not include samples collected in the presence of proteasomal or lysosomal inhibitors, it is likely that hypoxia-induced ubiquitination of selected proteins that target those proteins for degradation are not detected by our study. However, the current study design, by permitting identification of non-degradative hypoxia-induced, ubiquitin-mediated protein modifications, emphasizes the abundance of targets in pathobiologically relevant pathways that undergo UPS-mediated modification. As demonstrated by the analysis of protein levels of four different proteins that had changes in ubiquitination (Fig. 6), ubiquitination impacts more than just protein levels, and we postulate that non-proteasomal ubiquitin-mediated modifications can induce functional alterations in their protein targets. The participation of some of these proteins in multiple pathogenic pathways (Fig. 4a and Table 2) provides additional information to identify high-value targets for additional investigation. STRING analysis revealed direct interaction between 12 of the 17 proteins that function in three or more pathways (Fig. 4b). The central position of these ubiquitin-altered proteins within PH-related pathways suggests they may act as signaling nodes within more complex networks. Peptides for three of these interacting proteins were found to include other modifications such as oxidation (Cav1 and Ilk; indicated by OX superscript) and carbamidomethylation (RhoA; indicated by CM superscript) (Table 2). These findings suggest that ubiquitination may represent a single component of complex post-translational modifications that modulate protein activity and cellular responses to stimuli.

Another limitation of our study relates to the use of a rodent model of PH. It is well established that chronic hypoxia exposure in rodents does not produce the severe obliterative vasculopathy seen in patients with Group 1 PH, pulmonary arterial hypertension (PAH), and in patients with advanced stages of other groups of PH. In addition, PH is subdivided by the World Health Organization (WHO) into five subgroups, each associated with distinct as well as shared pathobiological mechanisms of disease. Exposure to chronic hypoxia is most analogous to Group 3 PH associated with chronic lung disease and hypoxemia.[82] Therefore, the findings in the present study may be most relevant to Group 3 PH and less relevant to other types of PH. While examination of the ubiquitin proteome in human lung samples will be important, human samples from PH patients, particularly from those with Group 3 PH, are precious and hard to come by. Although several targeted therapies have been developed for the treatment of Group 1 PH,[83–85] surveillance data suggest that worsening mortality and hospitalization rates among PH patients in this country are seen predominantly in patients with WHO Groups 2–5.[1] Thus, despite existing limitations in these animal models of PH, the examination of hypoxia-induced PH can inform and facilitate urgently needed novel therapeutic approaches for Group 3 and other types of PH.

Finally, as illustrated by our analysis of mouse lung and pulmonary artery proteasome activity, the specific tissue compartment examined can have a significant impact on studies examining UPS activity. While examination of human lung and pulmonary artery tissue from subjects with and without various types of PH will provide the ideal tissues for future studies, the scarcity of these samples will continue to present challenges to the field. In addition, our study was not designed to determine mechanistic roles for specific ubiquitinated proteins in PH pathogenesis. However, the enrichment of these proteins within pathways that play established roles in PH pathogenesis suggests that many of these post-translational protein modifications meaningfully contribute to the origins of this disorder and can provide fertile areas for future investigation.

It is well established that the UPS plays an important role in dysregulated cancer cell proliferation,[61,62] leading to the application of proteasomal inhibitors in the clinical management of selected malignancies.[8] Previous studies have suggested similarities between cancer biology and dysregulated pulmonary vascular cell proliferation in PH.[86,87] Not surprisingly, a growing number of studies support that the application of proteasomal inhibitors in experimental PH, as in cancer biology, can attenuate various features of the disorder.[4,12,14,15,70] The current study strives to extend this field by providing a more comprehensive analysis of protein ubiquitination in PH. Our results define a large number of proteins with significant changes in protein ubiquitination after hypoxia, many of which regulate important cellular functions that are dysregulated in PH, including cell proliferation. Continued investigation in this area may allow more specific interventions targeting UPS-modifying proteins, such as individual E3 ligases, deubiquitinases, or ubiquitin editing enzymes. Our results provide novel evidence for broad alterations in the lung ubiquitin proteome that can serve to focus and inform future studies of PH pathogenesis and therapy.

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Declaration of conflicting interests

The authors declare that there is no conflict of interest.

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