Published in final edited form as:

J Proteome Res. 2018 August 03; 17(8): 2623–2634. doi:10.1021/acs.jproteome.8b00086.

Multifunctional Activity-Based Protein Profiling of the Developing Lung

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Abstract

Lung diseases and disorders are a leading cause of death among infants. Many of these diseases and disorders are caused by premature birth and underdeveloped lungs. In addition to developmentally related disorders, the lungs are exposed to a variety of environmental contaminants and xenobiotics upon birth that can cause breathing issues and are progenitors of cancer. In order to gain a deeper understanding of the developing lung, we applied an activity-based chemoproteomics approach for the functional characterization of the xenometabolizing cytochrome P450 enzymes, active ATP and nucleotide binding enzymes, and serine hydrolases using a suite of activity-based probes (ABPs). We detected P450 activity primarily in the postnatal lung; using our ATP-ABP, we characterized a wide range of ATPases and other active nucleotide- and nucleic acid-binding enzymes involved in multiple facets of cellular metabolism throughout

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Author Contributions

E.G.S., C.A., A.T.W., and J.N.S. designed the experiments. N.C.S. extracted mouse lung tissues. E.G.S. and R.F.V. prepared probe-enriched and global peptides. A.K.S. performed the LC-MS proteomics. E.G.S. analyzed the proteomics data. J.P.C. and M.C.L. performed the RNA in situ hybridization experiments and analysis. E.G.S., C.A., and A.T.W. wrote the manuscript. All authors have given approval to the final version of the manuscript.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.jproteome.8b00086](https://doi.org/10.1021/acs.jproteome.8b00086).

Supporting Table S1. DAVID enriched ATP-ABP targets (XLSX)

Supporting Table S2. DAVID enriched global proteins with nucleotide-binding (XLSX)

Supporting Table S3. All identified proteins from global analysis (XLSX)

Supporting Table S4. **FP-2** serine esterase and hydrolase targets (XLSX)

Supporting Table S5. **P450-ABP** cytochrome P450 targets (XLSX)

Supporting Table S6. Results of DAVID Functional Annotation Analysis (XLSX)

The authors declare no competing financial interest.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner⁴⁴ repository with the data set identifier PXD008836 and DOI: [10.6019/PXD008836](https://doi.org/10.6019/PXD008836).

development. ATP-ABP targets include kinases, phosphatases, NAD- and FAD-dependent enzymes, RNA/DNA helicases, and others. The serine hydrolase-targeting probe detected changes in the activities of several proteases during the course of lung development, yielding insights into protein turnover at different stages of development. Select activity-based probe targets were then correlated with RNA in situ hybridization analyses of lung tissue sections.

Graphical Abstract

Keywords

lung development; activity-based protein profiling; chemoproteomics

INTRODUCTION

Lung diseases and disorders are a leading cause of mortality in infants in the United States.¹ Deaths due to lung disease in infants are primarily caused by defects resulting from premature birth including apnea, pneumonia, respiratory distress syndrome, and others. Lung diseases affecting individuals at different developmental stages include chronic obstructive pulmonary disease, asthma, and cystic fibrosis. A deeper understanding of enzyme activities during lung development will offer insight into key functions and functional responses that will inform an improved predictive understanding of the human lung.

Methods to measure gene expression, both on the transcriptional and translational levels, have proven effective in revealing cellular mechanisms. However, due to post-transcriptional and post-translational regulatory mechanisms, global methods to measure expression often fail as consistent indicators of enzymatic activity.² Thus, to investigate important mechanisms of lung development, we employed an activity-based chemo-proteomics approach that allows for the characterization of active enzymes of specific functionalities. This approach utilizes small molecule inhibitors that facilitate activity-dependent irreversible binding to enzyme(s) of desired functionality and enrichment, and identification of targeted enzymes by LC-MS based proteomics. Enrichment is accomplished through the addition of an alkyne moiety enabling click chemistry mediated attachment of a biotin molecule followed by streptavidin enrichment.³ In this study, we utilized five such probes previously synthesized and validated to specifically measure the activity of target enzyme classes. We

chose these probes to maximize the scope of our functional characterization to include enzymes we hypothesized to be highly important in lung development. The enzyme activities we sought to measure included xenobiotic metabolism, enzymes involved in the cell cycle, protein biosynthesis, cell:cell adhesion, and many others. In addition, we sought to add to the current knowledge and research concerning enzymes with potential involvement in lung surfactant metabolism and their roles in lung development. In our study, we extracted protein and conducted activity-based and global proteomics analysis on mouse lung lysate (S9 fraction) from gestational day (gd) 17, and postnatal days (pnd) 0, 21, and 42; representing the canalicular (gd17), early alveolar (pnd0) and late alveolar (pnd21) stages of lung development and the adult lung (pnd42). Validation of proteomics results from enzymes showing significant changes throughout development was conducted by RNA in situ hybridization on tissue slices from developing lungs as both an alternative measurement of expression as well as an investigation of enzyme localization within the tissue.

Cytochrome P450 monooxygenases are a family of phase I drug metabolizing enzymes that catalyze the NADPH-dependent addition of an oxygen molecule to many pharmaceutical drugs, environmental pollutants, natural dietary products, and other xenobiotics. The added oxygen molecule typically generates a more polar, water-soluble, and reactive product. This increased solubility and reactivity helps facilitate expulsion and/or phase II metabolism, which primarily involves conjugation. While the lung displays lower xenometabolizing activity than the liver,⁴ it has been shown to aid in the metabolism of a variety of xenobiotics either present in the air, or xenobiotics that are not neutralized and excreted during first pass liver metabolism.⁵ Due to the significant contribution of the lung in metabolism of inhaled environmental pollutants and in secondary drug metabolism, deficiencies in P450 activities in the developing lung could lead to increased susceptibility to toxic effects of xenobiotic exposure in distinct developmental stages. To target active cytochromes P450 in lung lysate, we used a multiprobe approach involving the use of probes, **2-EN**, **ATW8**, and **ATW12**, to increase coverage of the diverse P450 enzyme family. The probes are derivatives of irreversible small molecule inhibitors that, upon enzyme binding, are catalytically transformed into reactive electrophiles that bind nucleophilic amino acid residues in close proximity. These probes have previously been used, both individually and in combination, to target active P450s in murine and human tissues.⁶ Recently, this multi-P450 probe approach was successfully used to study ontogeny changes in active cytochromes P450 in human liver.

To target active nucleotide-binding enzymes in lysate, we used **ATP-ABP**.⁷ This probe contains two reactive acyl phosphate groups that label active site lysines of ATPases and nucleotide-binding proteins. Each reactive acyl phosphate group contains an alkyne handle to facilitate the addition of a reporter molecule for protein identification. This activity-based probe has previously been used to investigate the activity of mitochondrial kinases, NAD/FAD binding, and nucleic acid binding enzymes.⁷ This probe offers insight into central metabolic pathways, cell signaling, and oxidoreductase activity of NAD/FAD binding enzymes.

While **ATP-ABP** offers insight into activity of many enzymes involved in central carbon metabolism, **FP2** measures the metabolism of peptides and proteins through serine hydrolase activity. **FP2** can be indicative of protein turnover, and has been used previously to measure serine protease activity in murine tissue lysates.⁸ The probe functions as an irreversible inhibitor of serine hydrolases and features an alkyne handle for the attachment of a reporter group for enzyme identification. Serine proteases are known to play key roles in the metabolism of pulmonary surfactant,⁹ a mixture of lipids and proteins that prevent alveolar collapse to ensure proper lung function.

METHODS

Animals

All mice in proteomics experiments were B6129SF1/J $\times$ 129S/SvImj crosses. Female B6129SF1/J and male 129S1/SvImj mice were purchased from The Jackson Laboratory. Upon arrival, mice were given 7 days to acclimate before being subject to experimental procedures. Mice were housed with a 12 h light/ dark cycle. Mice had ad libitum access to standard lab chow and water (PMI Nutrition International Certified Rodent Diet 5002). Roughly half of the crosses of B6129SF1/J $\times$ 129S/SvImj are Ahr (aryl hydrocarbon receptor) responsive. Genotyping of offspring was conducted as previously described to determine Ahr responsiveness¹⁰ Only lungs of AhR responsive mice from the cross were used for these studies. All animal protocols were approved by the Institutional Animal Care and Use Committee at Pacific Northwest National Laboratory.

Preparation of Lung Lysate S9

Fetal and adult lung tissue was shredded in a sucrose (250 mM) in PBS solution (pH = 7.4) using a tissue tearor. Following this, tissue was transferred into a glass dounce homogenizer. Tissues were homogenized with 15 pulses using A and B pestles in sequence. Lysate was centrifuged at 10 000g for 25 min, and the supernatant S9 fraction was collected. Six total lungs from each developmental stage were homogenized. To have necessary proteome content for probe labeling experiments, samples were combined yielding 3 biological replicates. Protein concentration in the replicates was determined via the BCA assay.

Preparation of Global Peptides

Unenriched lung lysate S9 fractions from all samples were normalized to 100 μ L 0.6 mg/mL protein using a bicinchoninic acid assay (BCA Protein Assay Kit, ThermoScientific). Urea (8M) was added to all samples. Samples were reduced by incubating at 60 °C for 30 min with DTT (500 mM). Samples were then alkylated using IAM (40 mM) and incubated at 37 °C for 60 min. Trypsin was added at a 1:50 ratio of trypsin to protein and digestion was carried out for 3 h at 37 °C. Digested peptides were washed using C18 SPE columns, which were first conditioned with MeOH, equilibrated with TFA (0.1%). Samples were washed with H₂O:ACN (95:5) with TFA (0.1%), and eluted with H₂O:ACN (80:20) with TFA (0.1%). Eluted peptides were dried by speed vacuum and reconstituted in NH₄HCO₃ (25 mM). Peptides were normalized to 0.1 mg/mL for analysis.

Probe Labeling and Click Chemistry

All samples were normalized to 1 mg/mL protein content. Each sample was transferred into 500 μ L aliquots (4 total per replicate). Four probe conditions were used: DMSO only control, **P450-ABP** mixture (containing 2EN (20 μ M), ATW8 (20 μ M), and ATW12 (20 μ M)), **ATP-ABP** (20 μ M), and **FP2-ABP** (20 μ M). Three replicates from each developmental stage were incubated with probe or an equal volume of DMSO vehicle at 37 °C for 60 min, shaking at 1000 rpm. The requisite P450 cofactor, NADPH (2 mM), was also added to **P450-ABP** labeling reactions. Following incubation with probe(s), the click chemistry reaction was initiated and samples were incubated at room temperature in the dark for 1 h. Click chemistry reagents were individually added in the following order: biotin-azide or rhodamine-azide (60 μ M) (for streptavidin enrichment or SDS-PAGE analysis, respectively), sodium ascorbate (10 mM), tris(3-hydroxypropyltriazolylmethyl)amine (2 mM), and copper sulfate (4 mM). Following click chemistry, proteins were precipitated by adding cold MeOH and placed in a –80 °C freezer for 1 h. Samples were centrifuged at 14 400g for 10 min and supernatant was removed, leaving the precipitated protein pellet. SDS (1.2%) in PBS was added to each sample, and protein was resolubilized by sonicating with 4 $\times$ 1 s pulses at 60% amplitude with a probe ultrasonicator with sample heating at 95 °C for 5 min. All samples were then centrifuged at 6000g for 5 min at rt, and the supernatant containing resolubilized protein was collected.

Streptavidin Enrichment

Probe labeled proteins appended to biotin were enriched using streptavidin agarose beads (ThermoScientific). 100 μ L of streptavidin agarose beads were prewashed 2 $\times$ with SDS (0.5%) in PBS (1 mL), 2 $\times$ with urea (1 mL, 6M) in NH_4HCO_3 (25 mM), and 4 $\times$ PBS (1 mL), under vacuum using fritted columns (BioRad). Washed agarose resin was transferred to cryovials with PBS. Protein samples were then added to the cryovials with the resin. Bead/protein mixture was incubated at 37 °C for 1 h, with rotation.

Following incubation, the beads were transferred into BioRad columns and were washed 2 $\times$ with SDS in PBS (0.5%, 1 mL), 2 $\times$ freshly prepared urea (1 mL, 6 M) in NH_4HCO_3 (25 mM), 2 $\times$ with Milli-Q water (1 mL), 8 $\times$ PBS (1 mL), 4 $\times$ NH_4HCO_3 (25 mM, 1 mL). Agarose resin was then transferred to low-binding tubes (Eppendorf) using two aliquots of PBS (500 μ L). The resin was centrifuged at 10 500g for 5 min at rt and supernatant was discarded, leaving the resin which was resuspended in urea (400 μ L, 6 M) in NH_4HCO_3 (25 mM). Proteins were reduced and alkylated on the agarose resin with TCEP (5 mM) incubation at 37 °C for 30 min followed by iodoacetamide (10 mM) incubation at 50 °C for 45 min (under foil). Beads were again transferred to BioRad columns and subjected to the following washes: 8 $\times$ PBS (1 mL), 4 $\times$ NH_4HCO_3 (1 mL, 25 mM). The resin was transferred to sterile, individually wrapped 1.5 mL tubes with NH_4HCO_3 (1 mL, 25 mM). The resin was then centrifuged at 10 500g for 10 min to remove supernatant. The resin was rewetted with NH_4HCO_3 (200 μ L, 25 mM). Proteins were digested on the resin using a 1:4000 ratio of μ g protein (at start of enrichment) to μ g Promega trypsin. Samples were digested at 37 °C overnight, with rotation.

Trypsinized peptides were centrifuged at 10 500*g* for 5 min. Peptide-containing supernatant was transferred to new individually wrapped tubes. NH_4HCO_3 (150 μL , 25 mM) was added to the resin again, the solution vortexed, and centrifuged at 10 500*g* for 5 min. Supernatant was transferred to corresponding tubes from the initial collection. Tryptic peptide samples were placed on a speedvac concentrator until dry. Peptides were resuspended using NH_4HCO_3 (40 μL , 25 mM) and placed on a thermal shaker at 37 °C for 5 min at 1000 rpm. Resolubilized peptides were transferred to ultracentrifuge tubes and centrifuged at 100 000*g* for 20 min. Twenty-five μL per sample was then placed in appropriate MS vials for LC–MS analysis.

SDS-PAGE Analysis

Probe labeled and rhodamine appended proteins were resolved via SDS-PAGE. Ten μg protein was added to each well of a 4–12% Bis-Tris gel (Invitrogen). Samples were run at 200 V, 35 mA for 45 min. Gels were imaged using a Typhoon FLA 9500 (General Electric) followed by total protein staining and imaging using GelCode Blue and GelDocEZ (BioRad Laboratories).

LC–MS/MS Analysis of Enriched Probe Targets

Probe-enriched peptides were analyzed using a Velos Orbitrap instrument according to the method outlined in Sadler et al.¹¹

Data Analysis of Enriched and Unenriched Peptides

Peptide data was analyzed using an AMT tag approach.¹² Peptide spectra was searched against the *Mus musculus* Uniprot¹³ protein database and was rescored using MSGF+.¹⁴ Peptides used in further analysis were filtered if they met the following three criteria: (1) protein count = 1; (2) MT Uniqueness 0.5; (3) MT FDR 1%. This resulted in a list of peptides whose relative abundances were log₂ transformed and normalized via linear regression. Proteins were rolled up to the protein level using Inferno.¹⁵ Five proteins were used for the Grubb's test with a *p*-value 0.05. Rolled up proteins with greater than 3 unique peptides were used in further analysis. Functional enrichment and clustering of proteins was conducted using DAVID.^{16,17} All known mouse protein IDs were used as the background across all DAVID functional enrichments.

RNA In Situ Hybridization of Lung Tissue Slices

High-throughput RNA in situ hybridization (HT-ISH) was based upon previously described techniques.^{18–20} Briefly, C57BL/6J gd16.5 and gd18.5 torsos, as well as OCT/PBS/ sucrose inflated pnd7 and pnd28 lungs, were embedded in ice-cold OCT/PBS/sucrose, frozen and kept as frozen blocks at –80 °C until sectioning. Blocks were sectioned at 20 μm on Leica CM3050S cryostats and placed onto Superfrost Plus slides. HT-ISH was performed using a Tecan EVO GenePaint liquid handling platform to detect specific RNA expression within sections of mouse lung used nonradioactive digoxigenin-(DIG-)labeled probes with several signal amplification steps, including tyramide. Probes were generated from DNA templates, 300–1000 bp long, and designed to be unique to the investigated gene. Probes were validated either by comparison to published results or by sequencing the DNA template. Tissue

section images were produced at 0.5 μm resolution using a Zeiss Axio Scan.Z1 slide scanner. All procedures followed BCM IACUC approved protocols.

RESULTS AND DISCUSSION

Fluorescence Gel Analysis of P450, ATPase, and Serine Hydrolase Activities in the Developing Lung

We commenced our multifunctional activity-based investigation of lung development through visualization of probe-targeted proteins via gel fluorescence. Protein normalized lung lysate from developmental stages gd17, pnd21, and pnd42 were labeled with **ATP-ABP**, **FP2**, or **P450-ABPs** (ATW8, ATW12, 2EN). Subsequent addition of a fluorescent rhodamine reporter using click chemistry, enabled fluorescence visualization of probe-labeled proteins by SDS-PAGE. Additionally, probelabeled proteins were also visualized by Coomassie protein staining and indicated that the fluorescence gel labeling is independent of protein abundance.

As expected, due to its previously demonstrated wide range of enzyme targets, fluorescence gel analysis of **ATP-ABP** shows a number of probe labeled bands from various molecular weights in gd17, pnd21, and pnd42 lung lysate S9. Decreases in several of the visible bands occur from gd17 to pnd21 and pnd42 samples. Fluorescence gel analysis of **FP2** probe labeling shows increases in labeling of a 52 kDa protein and decreased labeling of a 26 kDa protein when comparing gd17 and pnd21/pnd42 sample. Mammalian proteases range in molecular weight from as small as 20 kDa to as large as 400 kDa. Thus, the wide range of **FP2** labeled protein bands as apparent on electrophoretic gel is expected. Converse to the large mass range of proteases, cytochrome P450 enzymes have molecular weights ranging from around 50–60 kDa. No band in this range is seen in gd17 samples; however, a 52 kDa band appears at pnd21 and pnd42 (Figure 1B). Previous studies have shown that P450s emerge in postnatal stages of lung development, coinciding with the differentiation of Clara cells, where cytochromes P450 are primarily expressed.²¹

Multifunctional Activity-Based Chemoproteomic and Global Proteomic Analysis of the Developing Lung

The promising fluorescence gel results prompted us to enrich probe-labeled protein from lysate S9 from several distinct developmental stages including gestational day 17 and postnatal days 0, 21, and 42 for proteomic analysis. To supplement probe-enriched chemoproteomics from these samples, we also conducted global analyses on the same lysates.

We incubated gd17, pnd0, pnd21, and pnd42 murine lung S9 lysates with **ATP-ABP**, **FP2**, and **P450-ABPs**. Following this, we attached a biotin molecule to the clickable alkyne for streptavidin enrichment. Enriched proteins were trypsinized and LC-MS/MS analysis was conducted. Our chemoproteomics analysis identified 140 enzymes in DMSO only controls, 523 enzymes in **P450-ABP** labeled samples, 188 enzymes in **FP2** labeled samples, and 624 enzymes in **ATP-ABP** labeled samples. 550 proteins were identified from global proteomics analyses of unlabeled samples.

Protein IDs identified with >3 unique peptides from the distinct ABP analyses (i.e., **ATP-ABP**, **FP2**, **P450-ABP**) and their corresponding global analyses were subjected to a functional enrichment analysis using DAVID and UniProt Keyword (UP_KEYWORD) annotations. For **ATP-ABP**, probe-enriched proteins containing the UP_KEYWORD annotations “nucleotide-binding”, “NAD”, “FAD” were functionally enriched. 212 total protein IDs from both global and **ATP-ABP** data sets were functionally enriched. Of the 212 IDs, 10 IDs were unique to the global data set, 110 IDs contained data in both global and probe-enriched samples, and 92 were unique to the probe data. Similarly, in both global and **FP2** enriched data sets, protein IDs with serine esterase/hydrolase activities were functionally enriched. No IDs with known serine esterase/hydrolase activities were unique to the global data, 5 IDs with such activity contained data in both global and ABP samples, and 4 IDs were found only in **FP2** probe enriched samples. Lastly, 2 of 3 functionally enriched cytochrome P450 monooxygenases were found only in probe-enriched samples and 1 ID contained data in both probe-enriched and global proteomics samples. In total, probe-enriched proteomes enabled identification and expressional measurement of nearly 100% more enzymes with ATPase, serine hydrolase/esterase, and P450 activities over nontargeted global analyses. In addition, less than 5% of all IDs with probe-related functions were unique to the global samples (Figure 1C). These results demonstrate the utility of a multifunctional ABP approach to significantly increase detection and measurement of protein targets with a swath of functionalities.

To determine the proteins whose measured AMT abundances significantly changed from early to later stages of development, $\log_2$ fold change abundances of gd17/pnd42 for all functionally enriched probe and global proteomics samples were plotted with the $-\log_{10}$ significance values as determined via *t* test. 102 total proteins show decreased nucleotide-binding activity (F.C. ≥ 2 , $p \leq 0.05$) from gd17 to pnd42. Additionally, 22 proteins contained data from gd17 samples, but not pnd42 samples where nucleotide-binding activity was not detected for this subset of proteins. Conversely, 8 proteins had significant increased nucleotide-binding activity at pnd42 samples in comparison to gd17 samples as determined via fold change and *t* test. Two additional proteins were not detected in gd17, but were detected in pnd42. All three proteins with known P450 activity were found to have increased activity at pnd42 in comparison to gd17 with two of these proteins containing no detection in probe-labeled samples at gd17. Finally, one serine protease significantly increased throughout development and one significantly decreased (Figure 2A).

Albeit with far fewer protein IDs exhibiting nucleotide-binding, serine protease, and P450 monooxygenase functions, overall functional trends observed via global proteomics seem to follow a similar pattern observed in activity-based data. Alterations and responses in enzymatic activities are often mechanistically attributed to changes in protein expression (whether at the transcriptional or translation levels) or activity. The source of these changes in activity versus expression could be due to cofactor availability, post-translational modifications, etc. Changes in activity of the majority of our probe-targeted proteins within the data set can be attributed to concomitant changes in expression as measured by global proteomics. However, five proteins show significant changes in activity that cannot be attributed to expression. These proteins include two ATPases and three serine proteases

including lung surfactant convertase. These proteins exhibited no significant change in expression ($\log_2$ fold change GD17 vs PND42 between -1 and 1 and $p > 0.05$). Additionally, these proteins had a fold change activity as determined by probe labeling greater than $3\times$ versus the fold change of global protein expression (Figure 2B).

Peptides from 624 enzymes were identified in **ATP-ABP** labeled samples. Functional enrichment analysis of probe-labeled and global samples using DAVID revealed **ATP-ABP** labeled samples contained 163 nucleotide-binding proteins (26.1% of total proteins identified) compared to 97 (17.6% of total proteins identified) in global samples (Figure 3C). The majority of this discrepancy between global and probe analyses seems to be primarily due to ATP-binding activity, as little difference is seen between proteins with GTP-binding activity. **ATP-ABP** labeled samples also contained more NAD-binding enzymes (31) than the global samples (17). In total 202 nucleotide- and NAD/FAD-binding enzymes were found in **ATP-ABP** labeled samples, compared to 120 in global samples.

To investigate the breadth of diverse functions that the targeted nucleotide-binding proteins contain, a further functional analysis of these targets was conducted using DAVID. A wide variety of functions emerged including many proteins with transferase, oxidoreductase, kinase, helicase, aminoacyl t-RNA synthetase, protease, and other activities. Furthermore, many proteins known to be involved in Ubl conjugation, the cell cycle/ cell division, protein folding, lipid metabolism, glycolysis, etc., were identified in **ATP-ABP**-enriched proteomes (Figure 3A).

Nucleotide-Binding Activities of Housekeeping Enzymes

We conducted further analyses on the 202 nucleotide- and NAD/FAD-binding enzymes to determine between which developmental stages the majority of the changes in nucleotide-binding activity occurred. We sorted this large group of enzymes based on (a) where the greatest change in activity occurred for each protein (as measured by AMT abundances of probe-labeled samples), and (b) whether the abundance increased or decreased with development. We found that the majority of these enzymes decreased throughout development and the largest decreases occurred between the date of birth (pnd0) and 21 days following (Figure 3B).

Twenty-one enzymes involved in the cell cycle were functionally enriched using DAVID's functional annotation clustering feature. These targeted proteins include cyclin dependent kinases, DNA replication licensing factors, map kinases, septins, and others. Cyclin dependent kinases 1 and 2 were enriched in gd17 and pnd0 but were not detected in pnd21 and pnd42 lungs. An 8-fold decrease in CDK1 activity occurred from gd17 to pnd0. CDK2 was detected in gd17, pnd0 and pnd21 and showed a time dependent decrease in activity with 2.85- and 3.85-fold decreases between gd17/pnd0 and pnd0/ pnd21, respectively. All probe-targeted DNA replication factors (MCM2, MCM3, MCM5, and MCM6) showed decreased activity from gd17 to pnd42. MAP kinases, MK01 and MK03, showed decreased activity from gd17 to pnd42 with 9.73 and 2.8-fold decreases, respectively with the majority of the activity decrease occurring between pnd0 and pnd21. Septins (SEPT2, SEPT9, SEPT7, and SEPT11) all showed decreased activity comparing gd17 to pnd42 samples (Figure 4A). The apparent decreases seen in cell cycle-related enzymes with increasing

development time is in line with prior observations that the earlier stages of lung development are characterized by high proliferation rates.^{22,23}

A set of 20 enzymes with terms related to cell:cell adhesion were clustered together. This set of enzymes include two oncogenes, septins, heat shock proteins, translation initiation factors, and others. These enzymes showed an average fold change decrease from gd17 to pnd0 and a large decrease from pnd0 to pnd21 (Figure 4B). As with the other housekeeping enzymes detected in this study, the activity and expression decreased through development and reached a homeostatic balance in adulthood with minimal changes detected when comparing pnd21 and pnd42 developmental stages.

Thirty-one **ATP-ABP** targeted enzymes involved in protein biosynthesis were also decreased markedly over lung development, with the most profound differences seen between the pnd0 and pnd21 age groups. These targets include 16 tRNA ligases, the activities of which decreased considerably from pnd0 to pnd21. We hypothesize these decreases in activity of these protein biosynthetic enzymes occur as the lungs reach maturity and protein biosynthetic activity equilibrates (Figure 5A).

Enzymes involved in protein folding and related functions decreased from gd17 to pnd0 and again from pnd0 to pnd21. These enzymes include the t-complex protein and most of its subunits, heat shock proteins, an ABC transporter, and 3 MAP kinases (Figure 5B). The chaperone T-complex proteins are involved in many other processes including telomere maintenance, RNA and localization to Cajal bodies, etc. Cajal bodies reside in the nucleus and are important for RNA and protein processes related to development.²⁴ Decreases in activities related to these bodies should be expected as the murine lung reaches maturation.

Development of Xenobiotic Metabolizing Enzymes

Probe-mediated enrichment revealed three active P450 isoforms that were targeted by the P450 probe mixture (Figure 6). Due to the high reactivity of **P450-ABPs** upon bioactivation by P450 enzymes, many proteins were enriched. The majority of enriched enzymes lacked data in the DMSO only controls. As this probe combination has been previously validated to bind and report on *active* P450s, we searched for cytochrome P450 enzymes in our protein data. We found no P450 enzymes were enriched in corresponding no probe controls and only one P450 monooxygenase was detected in corresponding global samples.

P450 2f2 was enriched at pnd0, pnd21, and pnd42. P450 2f2 shows time dependent increases in activity; 11-fold from pnd0 to pnd21 and stays static from pnd21 to pnd42. This isoform is primarily found in the lung and liver. P450 2f2 has been identified as one of the contributors in benzene bioactivation in mouse and human lungs.^{25,26} The human P450 2f1 enzymes are known to metabolize 3-methylindole, found in cigarette smoke, to reactive toxic metabolites.^{27,28}

P450 2b10 activity was not detected until pnd21 and showed no increase in activity from pnd21 to pnd 42. P450 2b10 is primarily found in the lung and liver. This murine isoform, as well as cyp2b family members in rat and human lung, is known to contribute to styrene oxidation and subsequent cellular toxicity.²⁹ P450 2b enzymes are also known to bioactivate

polyaromatic hydrocarbons, such as dibenzopyrene, to oxidized carcinogenic metabolites.³⁰ Enzymes in this subfamily also are necessary for the metabolism and tumorigenesis of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a molecule found in tobacco.^{31,32}

P450 4b1 enzymes are preferentially expressed in lung ER and active enzymes are detected at gd17, pnd21, and pnd42. Detection in gd17 fetal samples may suggest a possible role in lung development. This enzyme has much higher expression at pnd21 and pnd42 time points with a 6 fold increase over gd17. P450 4b1 enzymes are known to bioactivate 3-methylindole, as well as the polyaromatic hydrocarbon 2-aminoanthracene into a carcinogenic metabolite.^{27,33,34} Due to the roles of each of the probe-enriched P450s in the metabolism of inhaled toxicants, an in depth investigation of P450 activity in the developing human lung is warranted. A human P450 activity profile similar to this study would provide important insights into the toxicity of many of these P450 oxidized environmental metabolites and the susceptibility of individuals to them based on their developmental stages.

Two flavin-dependent monooxygenases (FMO1 and FMO2) were enriched by **ATP-ABP**. Flavin-dependent monooxygenases are phase I drug metabolizing enzymes, that, similarly to cytochrome P450s, catalyze the oxidation of various endo- and xenobiotics. In contrast to cytochromes P450, FMOs use FAD as the electron-donating cofactor instead of NADPH. FMO1 was detected in 1 biological rep at pnd0 and all pnd21 and pnd42 replicates. FMO2 was detected at all developmental time points with time dependent increases in activity from gd17 to pnd42. Pnd42 abundances were 29 fold higher than gd17 samples.

Biotransformative CBR2 (carbonyl reductase 2) also showed time dependent activity increases from gd17 to pnd42, with a 42- fold increase in relative abundance. Carbonyl reductases catalyze the reduction of many carbonyl-containing compounds, including various xenobiotics. Furthermore, carbonyl reductases show glutathione binding activity and have high affinity for carbonyl-containing glutathione conjugates formed by glutathione transferases.^{35,36} CBR2 enzymes are thought to primarily be involved in the reduction of carbonyl-containing compounds in the lung, specifically.³⁷

ATP-ABP also facilitated the enrichment of three alcohol dehydrogenases: ADH1, ADHX, and AK1A1. Alcohol dehydrogenases function in the oxidation of alcohols to aldehyde or ketone products. ADHX is a class III alcohol dehydrogenase that is primarily involved in the dehydrogenation of long-chain primary alcohols, with little activity toward ethanol substrates. Notably, AK1A1 catalyzes the NADP(+) dependent *reduction* of various aliphatic and aromatic aldehydes, including polycyclic aromatic hydrocarbons.³⁸ This enzyme is known to have procarcinogenic effects and is known to activate trans diol products of P450 oxidized PAHs. Activities of these alcohol dehydrogenases (ADH1, ADHX, and AK1A1) showed 8-, 5-, and nearly 3-fold decreases in activity when comparing gd17 and pnd42 samples, respectively.

The nucleotide-binding activities of several aldehyde dehydrogenases, namely AL1A1, AL1A2, AL1A7, AL9A1, and ALDH2, were measured. AL1A1 activity was induced with development, with a significant 5-fold increase in activity when comparing gd17 and pnd42.

Conversely, AL1A7 showed a near 5-fold decrease when comparing the same stages. AL1A7 has been found to oxidize benzaldehyde, propionaldehyde, and acetaldehydes to their respective carboxylic acids.³⁹ These proteins have the ability to not only metabolize endogenous compounds but can also metabolize and/or bioactivate drugs and xenobiotics, making the development of these enzymes crucial for both normal metabolism and xenobiotic defense.⁴⁰

Induction of Vesicular Trafficking and Endosomal Transport

Enzymes with known involvement in vesicular trafficking and endosomal transport were significantly induced with development (Figure 2A). These enzymes include two EH-domain containing proteins with a likely role in membrane reorganization and tabulation,⁴¹ and may play a role in the secretion of lung surfactant. Both enzymes exhibited increased ATP-binding activity from gd17 to pnd0. This increase in activity was not due to induced expression as no significant change between these stages was detected in global analysis. The increases in activity seem to correlate with the known increase in lung surfactant secretion upon birth.⁴²

Development of Serine Hydrolase/Esterase Activities

Nine serine protease enzymes were enriched by **FP2** with four of those detected in global samples (Figure 7). Two of these enzymes, PPCE and PPGB (prolyl endopeptidase and carboxypeptidase C) showed high fold change decreases in activity from gd17 to pnd42. PPCE showed a 290-fold decrease in activity from gd17 to pnd42 and PPGB showed a 690-fold change decrease. The large decrease in PPCE activity is only coupled with a 4-fold decrease in expression while PPGB was not detected at all via global analysis.

Conversely, one serine protease, EST1C, showed a significant 10-fold increase when comparing gd17 and pnd42 samples. Again, unlike the vast majority of enzymes with both activity-based and expression data within our data sets, this drastic increase in activity is not coupled with any significant increase in expression. EST1C is known to be involved in the extracellular metabolism of lung surfactant and assists in the conversion of surfactant subtypes.⁹ Increases in the activity of this enzyme primarily occur between pnd0 and pnd21. We hypothesize this activity is likely induced shortly after birth when lung surfactant is initially secreted. We also detected a significant decrease in CES1D (carboxylesterase 1D) activity from gd17 to pnd0. Measured expression of this enzyme shows developmentally dependent increases from gd17 to pnd0 and pnd21 to pnd42. This protein exhibits lipase activity and catalyzes the hydrolysis of mono- and triacylglycerols, and thus may be important in lung surfactant metabolism.⁴³

To investigate a possible mechanism of these expression-independent changes in protease activity throughout development, we searched for endogenous protease inhibitors within our global analysis. Of the 10 serine protease inhibitors found, five showed significant increases in relative abundance and only one significantly decreased. The expression of these inhibitors thus correlates negatively with protease activity, where most proteases show decreases in expression, with only one increasing in activity. This data is suggestive of

protease inhibition as a possible mechanism behind the observed expression-independent changes in measured enzyme-specific protease activities.

In Situ Hybridization on Lung Tissue Sections

The previously described activity-based and global proteomics results suggest potentially significant developmental roles for the many enzymes showing large changes in activity and expression. These developmentally dependent enzymes comprise a broad range of function including cell cycle, protein biosynthesis and metabolism, cell:cell adhesion, xenobiotic metabolism, and others. To both complement and validate some of our findings, we conducted in situ hybridization on lung tissue sections from both fetal and adult mice. Two proteins involved in xenobiotic metabolism (*Fmo2* and *Aldh1a2*) and one serine hydrolase (*Ces1d*) were investigated using this method (Figure 8).

Results showed that *Aldh1a2* appears to have decreased expression through development as both stages of fetal tissue show increased staining in comparison to the postnatal sections. Additionally, both postnatal stages showed no visible differences compared to controls indicating expression below the limits of detection. This correlates well with our proteomics results where *Aldh1a2* (*AL1A2*) shows a decreasing trend from fetal to postnatal samples in both expression and activity. In situ results indicate *Fmo2* has strong expression across all developmental stages. While the proteomics results indicated greatly increased expression and activity of *Fmo2* through development, only minimal differences were readily apparent by in situ between developmental stages. These results seem to contradict our proteomics results; however, a closer investigation at the localization of *Fmo2* expression revealed large development-dependent increases in expression within the airways of the lungs. Both fetal stages exhibited no specific localization, with widespread and relatively uniform expression throughout the tissue. In contrast, both postnatal stages show increasingly specific localization to airways. These increases in expression within the airways may account for the large activity and expression activity increases measured by proteomics. These in situ results, combined with proteomics results, further suggest an important role of *Fmo2* in the metabolism of inhaled toxicants/ xenobiotics upon birth.

In situ hybridization results also showed that *Ces1d* expression progressively localizes to the airways with increased development. Both fetal lung tissue stages showed broad expression of *Ces1d* with expression becoming increasingly localized to the airways postnatally. These results, combined with the increased protein expression observed via global proteomics, provide further evidence of a potential role of *Ces1d* in lung surfactant metabolism.

CONCLUSIONS

Activity-based protein profiling has been repeatedly used to functionally characterize complex proteomes from diverse biological systems. In general, these probes have been used individually within perturbed systems. While this offers a more targeted approach than conventional global proteomics platforms, a significant portion of protein activity goes unmeasured. Here we show the utility of a multifunctional ABPP approach, using five different ABPs to measure functionality of P450s, proteases, and nucleotide-binding enzymes within the developing murine lung. To correlate proteomics results and provide

further physiological context, we performed RNA in situ hybridization on pre- and postnatal lung tissue sections to investigate both overall enzyme expression as well as localization.

ATP-ABP yielded many distinct targets from enzymes of various functionalities including enzymes with key roles in the cell cycle, protein biosynthesis and degradation, gene expression and regulation, carbon and lipid metabolism, and a variety of NAD and FAD binding enzymes including several involved in the metabolism of xenobiotics. Together, the **ATP-ABP** and **P450-ABPs** target a set of diverse phase I drug metabolizing enzymes. While the **P450-ABPs** mechanism of enzyme binding is due to a direct enzymatic action on the probes, creating a reactive intermediate that binds nucleophilic amino acids within the binding pocket, the **ATP-ABP** binds enzymes due to enzyme nucleotide binding capacity. Together, these probes target cytochrome P450s, flavin monooxygenases, alcohol dehydrogenases, and carbonyl reductases. The use of both of these probes in tandem may prove an effective method to report on the activity of phase I metabolism.

This approach offers an in-depth activity-based chemo-proteomic investigation into the developing murine lung. As expected, probe targets involved in cell cycle, protein biosynthesis and degradation, and gene regulation greatly decreased with lung maturity. Conversely, an enzyme specific upregulation of phase I metabolism was seen as the lung matured. Further studies using a combination of phase I and II drug metabolism ABPs could yield a more comprehensive and in depth look at xenobiotic metabolism in mammalian systems. Such studies would provide pharmacological and toxicological insights into the susceptibility of the developing lung to drugs and environmental toxicants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

Portions of this research were supported by grants from the National Heart Lung Blood Institute of NIH (U01 HL122703) and the National Institute of Environmental Health Sciences (P42 ES016465). Proteomic and chemoproteomic technologies used herein were developed in part by a grant from the National Institute for General Medical Sciences (P41 GM103493). RNA in situ hybridization was supported by a shared equipment grant from the NIH (S10 OD016167) to M.C.L. Work was performed in the W. R. Wiley Environmental Molecular Sciences Laboratory (EMSL), a DOE-BER national scientific user facility at Pacific Northwest National Laboratory (PNNL). PNNL is operated for the DOE by Battelle under contract no. DE-AC05-76RLO-1830.

ABBREVIATIONS

NAD	nicotinamide adenine dinucleotide
FAD	flavin adenine dinucleotide
LC-MS	liquid chromatography-mass spectrometry
DTT	dithiothreitol
IAM	iodoacetamide
TFA	trifluoro-acetic acid

ACN	acetonitrile
DMSO	dimethyl sulfoxide
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
NADPH	nicotinamide adenine dinucleotide phosphate
PBS	phosphate buffered saline
AMT	accurate mass and time
CDK1	cyclin dependent kinase 1
CDK2	cyclin dependent kinase 2
MAP	mitogen activated protein
MeOH	methanol
MK01	mitogen activated protein kinase 1
MK02	mitogen activated protein kinase 2
SEPT2	septin-2
SEPT9	septin-9
SEPT7	septin-7
SEPT11	septin-11
ABP	activity based probe
ADH1	alcohol dehydrogenase 1
ADHX	alcohol dehydrogenase class 3
AK1A1	alcohol dehydrogenase [NADP-(+)]
PAH	polyaromatic hydrocarbon
AL1A1	retinal dehydrogenase 1
AL1A2	retinal dehydrogenase 2
AL1A7	aldehyde dehydrogenase cytosolic 1
AL9A1	4-trimethylaminobutyraldehyde dehydrogenase
ALDH2	aldehyde dehydrogenase, mitochondrial
EH	Eps15 homology
EST1C	carboxylesterase 1C

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Figure 1.

(A) Overview of experimental processes and subsequent analysis and structures of the activity-based probes ATP-ABP, FP2, and P450-ABPs. (B) SDS-PAGE gel of in vitro probe labeled murine lung proteome using 10 μ M ATP-ABP, FP2, and P450-ABPs: 2EN, ATW8, ATW12. Following probe labeling, rhodamine azide was appended onto the probe:protein complexes via click chemistry and 10 μ g protein per lane was loaded onto the gel cassette followed by SDS-PAGE and fluorescence imaging of probe-labeled protein (top). Gels were then stained using GelCode Blue and total protein stain was imaged using Bio-Rad (bottom). (C) Comparative analysis of functional enrichment of identified proteins from probe-enriched murine lung versus corresponding global proteomes.

Figure 2.

(A) Volcano plots of activity-based (top) and global (bottom) log₂ fold change AMT abundances and significance (*t* test) of identified proteins from gd17 versus pnd42. Horizontal dotted line is plotted where $p = 0.05$. Vertical lines placed at fold change = 2. Red dots indicate FP-2 targets, blue indicates P450-ABP targets, and black shows ATP-ABP targets. (B) Log₂ fold change abundances of gd17/pnd42 from global vs ABP analysis. Selected serine proteases (top) and nucleotide-binding (bottom) proteins shown are those whose expression does not significantly change but activity is significantly altered ($p < 0.05$, F.C > 2).

Figure 3. (A) Representative UP_KEYWORDS enriched from nucleotide-binding proteins from ATP-ABP enriched lung proteome. (B) Heatmap of relative abundances of proteins from the DAVID functional enrichment of ATP-ABP lung samples. Protein IDs are grouped on the basis of largest abundance differences between developmental stage and whether this change is positive or negative (with increasing development). 1: largest change between gd17 and pnd0, negative; 2: between gd17 and pnd0, positive; 3: between pnd0 and pnd21, negative; 4: between pnd0 and pnd21, positive; 5: between pnd21 and pnd42, negative; 6: between pnd21 and pnd42, positive. (C) Comparative functional DAVID enrichment of nucleotide-, NAD-, FAD-, ATP-, GTP-binding proteins from ATP-ABP enriched and global lung proteome.

Figure 4.

DAVID functional annotation clusters (AC) from DAVID analysis of active nucleotide-binding proteins with significant decreases in relative abundances between gd17 and pnd42. Also included in the functional analysis are proteins with data in gd17 samples and without in pnd42 samples. Heatmaps of the DAVID analysis shown on bottom, average fold change vs gd17 of proteins within each individual cluster at each devo stage plotted as line graph on top. Results from clusters 4 (left) and 14 (right) shown. Error bars represent standard error of the mean.

Figure 5.

DAVID functional annotation clusters (AC) from DAVID analysis of active nucleotide-binding proteins with significant decreases in relative abundances between gd17 and pnd42. Also included in the functional analysis are proteins with data in gd17 samples and without in pnd42 samples. Heatmaps of the DAVID analysis shown on bottom, average fold change vs gd17 of proteins within each individual cluster at each devo stage plotted as line graph on top. Results from clusters 2 (top) and 3 (bottom) shown. Error bars represent standard error of the mean.

Figure 6. Heatmap depicting relative log₂ abundances of ATP-ABP and P450-ABP enriched proteins involved in xenobiotic metabolism. Log₂ abundances shown are scaled to the average of all abundance values within each individual protein ID. Gray blocks indicate lack of abundance data at those developmental stages.

Figure 7.

Heatmaps depicting log₂ fold change abundances (versus GD17) of serine protease expression via global analysis (left) and activity analysis (right) as well as expression of serine protease inhibitors (bottom). Gray blocks indicate lack of abundance data at those developmental stages.

Figure 8. Results from in situ hybridization indicating expression of Aldh1a2, Ces1d, and Fmo2 in pre- and postnatal lung tissue slices. “a” and “v” indicate airways and vasculature, respectively.