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Hepatic cytochrome P450 activity, abundance, and expression throughout human development

Natalie C. Sadler, Premchendar Nandhikonda, Bobbie-Jo Webb-Robertson, Charles Ansong, Lindsey N. Anderson, Jordan N. Smith, Richard A. Corley, Aaron T. Wright

Biological Sciences Division, Pacific Northwest National Laboratory, Richland, WA, 99352 USA (N.C.S., P.N., C.A., L.N.A., J.N.S., R.A.C., A.T.W.)

Computational and Statistical Analytics Division, Pacific Northwest National Laboratory, Richland, WA 99352 USA (B.J.W.R.)

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Corresponding Author:

Aaron T Wright

Aaron.Wright@pnnl.gov

902 Battelle Blvd

Box 999, MSIN J4-02

Richland, WA 99352

(509) 372-5920 (office)

(509) 371-7304 (fax)

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Alphabetical Listing of Abbreviations: ABP, activity-based probe; ABPP, activity-based protein profiling; AMT, accurate mass and time; DME, drug metabolizing enzyme; FDR, false discovery rate; GD, gestational day; LC-MS, liquid chromatography-mass spectrometry; MSGF+, mass spectra generating function plus; P450, cytochrome P450; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; SD, standard deviation

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ABSTRACT

Cytochrome P450s are oxidative metabolic enzymes that play critical roles in the biotransformation of endogenous compounds and xenobiotics. The expression and activity of P450 enzymes varies considerably throughout human development; the deficit in our understanding of these dynamics limits our ability to predict environmental and pharmaceutical exposure effects. In an effort to develop a more comprehensive understanding of the ontogeny of P450 enzymes, we employed a multi-omic characterization of P450 transcript expression, protein abundance, and functional activity. Modified mechanism-based inhibitors of P450s were used as chemical probes for isolating active P450 proteoforms in human hepatic microsomes with developmental stages ranging from early gestation to late adult. High-resolution liquid chromatography mass spectrometry was used to identify and quantify probe-labeled P450s allowing for a functional profile of P450 ontogeny. Total protein abundance profiles and P450 rRNA was also measured, and our results reveal life-stage-dependent variability in P450 expression, abundance, and activity throughout human development and frequent discordant relationships between expression and activity. We have significantly expanded the knowledge of P450 ontogeny, particularly at the level of individual P450 activity. We anticipate that these results will be useful for enabling predictive therapeutic dosing, and for avoiding potentially adverse and harmful reactions during maturation from both therapeutic drugs and environmental xenobiotics.

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INTRODUCTION

The expression and activity of drug metabolizing enzymes (DME) are major determinants of the overall pharmacokinetic differences observed between adults, children, neonates, and prenatal individuals (Hines and McCarver, 2002; Hines, 2008). Cytochrome P450s are heme-containing DMEs with oxidase activity, and are found at highest concentrations in the liver. This enzyme superfamily is responsible for the biotransformation of endogenous compounds, pharmacological agents, and environmental xenobiotics (Nelson et al., 1993). However, P450s can also metabolize pro-toxins to toxins resulting in detrimental health effects. A comprehensive understanding of P450 expression and activity throughout human development is needed to inform predictive therapeutic dosing, and enable the avoidance of potentially adverse and harmful reactions during maturation from both therapeutic drugs and environmental toxins (de Wildt et al., 1999; Hines, 2008; Myllynen et al., 2009; Lee et al., 2012).

Cytochrome P450 expression and activity varies dramatically through prenatal development and from neonate maturation to adulthood. Differences between growth stages in DME composition and function can lead to neonate and childhood toxicity due to inaccurate dosing and dangerous exposure (Lee et al., 2012), as exemplified in the classic historical case of adverse reactions of neonates to chloramphenicol when doses were extrapolated from adult levels (Myllynen et al., 2009). Variability in P450 activity can lead to severe pharmacological effects by altering drug disposition, resulting in drug underexposure, overexposure, or toxic drug interactions (Nebert, 2000; Blake et al., 2005). To account for life-stage-dependent variability in P450 expression throughout human development, P450s have been parsed into three broad classes: (1) those P450s that are expressed at highest levels in during the first trimester of gestation; (2) those with fairly constant expression from gestation through adulthood; and (3) those with

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negligible or low prenatal expression, which then increase through postnatal maturation (Hines, 2007; Hines, 2013). Herein, we correlate expression-based measurements to functional activity measurements of individual P450s in the primary xenobiotic metabolizing families 1-3, as well as other families associated with xenobiotic metabolism and steroidogenesis. Throughout human development complete voids of knowledge still exist for P450 ontogeny, in terms of both P450 expression and activity.

There is a critical paucity of data on the ontogeny of individual P450 isoforms through maturation due to the scarce availability of tissue samples, largely forcing the use of rodents for ontogeny studies. But, significant variability between species in the primary structure, function, and regulation of different P450 isoforms makes it difficult to extrapolate between species (Hines, 2008; Myllynen et al., 2009). Available human data has revealed a number of discrepancies leading to difficulties in characterizing P450 ontogeny, including: limited sample sets, disparate measurement techniques, high inter-individual variability observed between different samples sets, and large differences between rodent and human P450 cohorts (Hines, 2008).

Herein, we have sought to expand our knowledge of P450 expression and activity in the human liver from early gestation to late adulthood using classic techniques to measure gene and protein expression, and have also utilized a chemical probe approach, activity-based protein profiling (ABPP), to interrogate P450 activity. Activity-based protein profiling of P450s uses modified P450 mechanism-based inhibitors as probes to profile the functionally active cohort of enzymes directly in microsomes isolated from human liver tissues (Wright and Cravatt, 2007; Cravatt et al., 2008; Wright et al., 2009; Crowell et al., 2013; Ismail et al., 2013). The approach requires the catalytic activity of the P450s to form a covalent bond between P450 and probe, from which we identify the targeted proteins by liquid chromatography-mass spectrometry (LC-MS) based proteomics or fluorescent gel imaging. Together, our results provide a thorough

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analysis and comparison of P450 expression and activity profiles through human maturation.

MATERIALS AND METHODS

Reagents and Chemicals. miRNeasy Mini Kit (Cat. No. 217004), QuantiTect Primer Assays, and QuantiTect SYBR Green RT-PCT kit were obtained from Qiagen. TURBO DNA-free kit was purchased from Invitrogen. Materials for labeling and enrichment include NADPH tetrasodium salt (Enzo Life Science), streptavidin agarose resin (Thermo Fisher Scientific; 1–3 mg biotinylated BSA protein per mL resin), sequencing grade trypsin (Promega), Pierce BCA Protein Assay Kit (Thermo Scientific), and Bio-Spin chromatography columns (Bio-Rad, Hercules, CA).

Human Tissue Samples. All prenatal human liver tissue samples (de-identified) were obtained from the Birth Defects Research Laboratory tissue repository at the University of Washington. At time of collection, prenatal samples were split with some of the tissue placed in RNA Later (qRT-PCR analysis), and the remainder flash frozen (ABPP and proteomic analyses) and stored at -80°C. Additional human liver microsome samples obtained from a commercial source (XenoTech, LLC, Lenexa, KS), and reported by Smith et al. (Smith et al., 2011), were included in this study to extend the enzyme analyses through neonatal and adult developmental ages (sample metadata can be found in *Supplemental Dataset 1*; available metadata for each sample is rather disparate, and therefore we chose not to include it in our statistical analyses or data interpretation). PNNL's Institutional Review Board approved all sample transfer procedures. Total number of prenatal liver samples is 30, ranging from gestational day (GD) 87-140; five samples are from late first trimester, and 25 samples are from early to mid-second trimester of development. Additional samples we evaluated are

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neonate/infant (age <1 yr; n=6), juvenile (age >1 yr and <18 yr; n=10), and adult (age >18 yr; n=9).

Microsomal Preparation. Liver tissues were processed according to prior methods with some minor changes (Wright and Cravatt, 2007). Tissues were rinsed with ice-cold 1.5% KCl, finely minced with a tissue tearor homogenizer (Biospec) for five 1 sec pulses and dounce homogenized with both a loose and tight fitting pestle in 250 mM sucrose in EDTA-free PBS buffer (~2 mL per tissue). The samples were then centrifuged at 10,000 x g for 20 min at 4 °C to remove cellular debris, nuclei and mitochondria. The supernatant was then centrifuged at 100,000 x g for 90 min at 4 °C to separate the microsome from the cytosol. Microsomes were resuspended in 250 mM sucrose in PBS buffer with minimal dounce homogenization, and protein concentrations were determined by a BCA assay. All samples were stored at -80 °C until use.

Activity-Based Protein Profiling (ABPP). ABPP utilizes chemical probes derived from aryl- or aliphatic-alkyne mechanism-based inhibitors of P450 enzymes, which directly report upon the activity of P450 enzymes in subcellular fractions, whole cells or organisms (Wright and Cravatt, 2007; Cravatt et al., 2008; Hollenberg et al., 2008; Wright et al., 2009; Crowell et al., 2013). Herein, we utilize a multiplexed labeling approach in which an arylalkyne probe (**P450-ABP1**)(Wright and Cravatt, 2007) and an aliphatic alkyne probe (**P450-ABP2**) (Wright et al., 2009) are added simultaneously to human prenatal tissue microsome samples (**Figure 1**). Multiplexed ABPP yields broad coverage of target enzyme families (Wiedner et al., 2012); this is particularly true in the P450 superfamily, in which individual P450 isoforms often display broad substrate specificity (Crowell et al., 2013). Aliphatic and arylalkynes have been demonstrated to inhibit P450s from all major detoxifying P450 subfamilies (Wright et al., 2009). In the

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presence of NADPH functionally active microsomal P450s directly oxidize the probes yielding reactive ketene moieties, which subsequently react irreversibly with the functional P450 (Wright and Cravatt, 2007; Wright et al., 2009; Crowell et al., 2013). Each probe also contains a second alkyne group that serves as a reactive handle for copper-catalyzed azide-alkyne cycloadditions (click chemistry). Following probe labeling of P450s click chemistry is used to append fluorescent reporters for gel imaging or biotin for enrichment and LC-MS analyses of probed enzymes (Speers et al., 2003; Wright and Cravatt, 2007).

Human liver microsomal proteome samples (1 mg/mL protein concentrations, 800 µg total protein) were treated with a mixture of activity-based probes (20 µM each of **P450-ABP1** and **P450-ABP2**). To probed samples was added NADPH (0.63 M), a requisite P450 cofactor for activity, and samples were incubated at 37 °C for 60 min in the dark. Control samples were prepared by heat-shock pretreatment of the microsome samples (98 °C, 10 min) prior to addition of ABPs and NADPH. Following ABP incubation, the samples were reacted with a biotin-azide (30 µM) tag under click-chemistry conditions as described previously (Wright and Cravatt, 2007; Crowell et al., 2013). Prior to enrichment of probe-labeled proteins, sample protein amounts were normalized to 350 µg protein. Probe-labeled proteins were enriched on streptavidin resin, reduced with tris(2-carboxyethyl)phosphine (TCEP, 2 mM), and alkylated with iodoacetamide (IAA, 4 mM). Proteins were digested on-resin with trypsin, and the resulting peptides were collected and analyzed by LC-MS as described previously (Crowell et al., 2013).

Proteomics Data Analysis. Generated MS/MS spectra were searched using the mass spectral generating function plus (MSGF+) algorithm against the publicly available *Homo sapiens*-translated genome sequence (www.uniprot.org; Sept. 2013 collection)(Kim et

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al., 2008). Identified peptides of at least six amino acids in length having MSGF scores $\leq 1E-10$, which corresponds to an estimated false discovery rate (FDR) $< 1\%$ at the peptide level, were used to generate an accurate mass and time (AMT) tag database (Zimmer et al., 2006). Matched peptide features from each LC-MS data set were then filtered on a FDR of less than or equal to 5% using the Statistical Tools for AMT tag confidence metric (Stanley et al., 2011). When a peptide is identified by the AMT tag approach, its relative abundance can be determined by calculating the area under the curve of the peptide ion peak in the MS measurement. Relative peptide abundance measurements in technical replicates were normalized to the data set with the least information using linear regression in DANTE (Polpitiya et al., 2008). Missing peptide abundance values were imputed with half of the minimum peptide abundance value across the entire dataset. Peptide abundance values were then rolled up to proteins using RRollup (Polpitiya et al., 2008); a minimum of three peptides was required for the Grubb's test, with a p -value cutoff of 0.05. Only peptides unique in identifying a single protein were utilized to estimate protein abundances. Additionally, due to low probe-dependent labeling of P450 activity, enrichment of active P450s resulted in low abundance measurements in the MS, therefore proteins represented by a single unique peptides were permitted. To identify a protein as specifically labeled by the ABPs, we required the following criteria: (1) peptide measurements for a protein exhibit a significant difference comparing ABP-labeled with NADPH to ABP-labeled heat-shock controls ($p < 0.05$); and, (2) the protein exhibits ≥ 4 -fold more abundance in the ABP-labeled with NADPH sample relative to the heat-shock controls.

Proteomics Statistical Analysis. The primary goal of the statistical analysis was to compare the average expression of the prenatal (trimester one (TM1) and trimester two (TM2)) sample groups to the average expression of the neonate/infant, juvenile, and

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adult sample groups. To account for missing data, two univariate statistical tests were performed on each protein across all age ranges, 1) a simple two sample t-test, and 2) a g-test (Webb-Robertson et al., 2010). The two-sample t-test identifies proteins that have a quantitative significant log₂ fold change difference between the two groupings and the g-test identifies proteins that have a significant difference in the presence or absence of identifications between the groups. In particular, missing data may be either censored (below the limit-of-detection of the instrument) or missing at random. The g-test evaluates the null hypothesis that the data is missing at random and a significant result indicates that the data is censored from one group or the other. All p-values with both the t-test and g-test evaluations were corrected with a Bonferroni correction to account for the multiple tests being performed. A Bonferroni was selected both because it is very stringent and the number of tests performed is relatively small (<30 in either the global or ABPP datasets) (Webb-Robertson et al., 2015).

The evaluation of the global data found 17 proteins with a significant increase from the prenatal group to the postnatal group with a Bonferroni correction and an additional three without the correction (*Supplemental Table 2*). Of these, six were significant only based on the g-test (censoring), nine were significant only based on log₂ fold change, and the remaining five were significant by both analyses and with confirmation of an increase in abundance by both tests. The global data had three proteins that decreased with a Bonferroni correction and an additional one without the correction; three with a quantitative change and one with a qualitative change. *Supplementary Dataset 2* gives the Bonferroni corrected p-values, the percentage of the data within each group observed for significant g-test results and the log₂ fold change for significant quantitative results.

The evaluation of the ABPP data found 11 total proteins with a significant increase from the prenatal group to the postnatal group with a Bonferroni correction and

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an additional two proteins significant prior to the correction. All of the significantly increasing proteins were identified via a g-test. There were three significantly decreasing proteins with a Bonferroni correction, and an additional two proteins with no correction. Of these five, two were qualitative differences and three were based on fold changes (see *Supplemental Table 3* and *Supplementary Dataset 3*).

Global Proteomic Analysis of Microsomes. Two replicates of microsomal lysate (100 µg) were denatured, solubilized and reduced with 8 M urea, 1% CHAPS, and 5 mM dithiothreitol (DTT) respectively, and incubated at 60 °C for 30 min. Samples were then alkylated with 40 mM iodoacetamide (IAA) at 37°C for 60 min. Each sample was diluted 8-fold with NH₄HCO₃ (100 mM, pH 8.4) to reduce salt concentration. CaCl₂ (1 mM) was added to the diluted samples, and proteins were digested using sequencing grade trypsin (Promega) at a ratio of 1 unit of trypsin per 50 units of protein for 3 h at 37 °C. Solid phase extraction was performed on digested samples using Supelco LC-SCX columns. Samples were acidified with 20% formic acid to achieve 1% final concentration. Columns were conditioned with 1 mL aliquots of MeOH (2x), 1 mL aliquots of 0.1% trifluoroacetic acid (TFA) in water (2x), and 1 mL aliquots of 1% TFA in water (2x). Samples were then loaded slowly and washed with 1 mL aliquots of 0.1% TFA in MeOH (4x). Samples were eluted with 1 mL of 5% NH₄OH in 30% MeOH. Sample volumes were reduced by vacuum centrifugation until dry, and reconstituted in 2% acetonitrile in water and centrifuged at 10,000 x g for 5 min to pellet column residue. Peptide concentrations were determined via the BCA assay. Sample protein concentrations were normalized to 0.25 µg/µL and characterized by LC-MS. Data analysis was consistent with the methods listed above, including statistical confidence metrics.

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Quantitative Reverse Transcription-Polymerase Chain Reaction. All human prenatal tissue samples were removed from RNeasy lysis solution. Tissues were diced into small pieces, and the pieces were transferred into 25 mL tubes containing the QIAzol reagent. Using a tissue tearer homogenizer the liver pieces were shredded fully and completely using a small gauge generator. The samples were homogenized at least 3x for 30-45 sec. Samples were kept on ice to prevent overheating and RNA degradation. Chloroform (0.20 mL) was added into each sample per 1 mL QIAzol lysis reagent, and then the samples were vigorously shaken by hand for 30 sec and the samples were centrifuged at 10,000 x *g* for 20 min at 4 °C. Further total RNA was extracted using the RNeasy mini kit (Qiagen) according to the manufacturer's directions. Total RNA purity and concentration was determined by NanoDrop. Genomic DNA was removed from the RNA samples by DNA-free™ DNase treatment (Invitrogen). The accession numbers of orthologous CYP genes were obtained from Choudhary et. al. (Choudhary et al., 2005). Primers (see *Supplemental Table 1*) for amplification of genes were obtained from Qiagen (Premade QuantiTect Primer assays).

A QuantiTect SYBR Green RT-PCR kit (Qiagen) was used for quantitative reverse-transcription polymerase chain reaction (qRT-PCR) following the manufacturer's recommendation. qRT-PCR was performed on an ABI Prism 7700 sequence detection system (Applied Biosystems). qRT-PCR was carried out with the QuantiTect SYBR Green RT-PCR master mix (Qiagen) using 50 ng RNA in a 25 µL final reaction mixture (reverse transcription 30 min at 50 °C; PCR initial activation 15 min at 95 °C). The average threshold cycle (Ct) for each gene was determined from duplicate reactions and normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin (Barber et al., 2005). Data was analyzed by taking the difference between mean threshold of qRT-PCR cycle values for individual P450 genes and the endogenous GAPDH control (*Supplemental Datasets 4, 5, and 6*).

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RESULTS

Transcriptional Ontogeny of P450 Expression in the Developing Prenatal Liver. Gene expression of P450s was measured in human prenatal livers. We were primarily focused on characterizing the expression of P450 genes representing the primary drug/xenobiotic metabolizing and steroidogenesis families, which are important for predicting chemicals prenatal individuals may be particularly sensitive to and developmental functions associated with steroid syntheses, respectively. The relative levels of P450 gene expression as compared to an internal control (GAPDH) are shown for individual prenatal livers in **Figure 2**. The overall pattern of P450 expression is fairly similar in the panel of prenatal livers. β -actin was also used as a second internal control and showed nearly identical results in comparison to the GAPDH. β -actin results are shown in *Supplementary Dataset 3*.

Proteomic Profiling of P450 Protein Expression in the Liver from Gestational through Adult Development. Global quantitative proteome profiling was performed to characterize individual P450 abundance in each of the prenatal liver samples. Thirty P450s were identified throughout the panel of tissues regardless of age, with 15 from the primary drug metabolizing subfamilies 1, 2, and 3, and 15 others with minor roles in drug metabolism and/or considerable roles in steroidogenesis. Distinctive patterns of protein expression emerge that are consistent with class-based organization of hepatic drug metabolizing enzymes (**Figure 3**). These proteins were grouped by activity patterns of LC-MS identification and loosely fall into the previously described P450 ontogeny classifications and include class 1 (those with a significant decrease in postnatal samples) 3A7, 7B1, 19A1, 51A1; class 2 (consistent from gestation through adulthood) 1A1, 2W1, 17A1, 39A1; and class 3 (significant increase in postnatal samples) 1A2,

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2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 2J2, 3A4, 3A5, 4F2, 4F3, 4F11, 4F12, 4F22, 4V2, 8B1, 20A1, 27A1.

Activity-Based Proteomic Profiling of P450 Function in the Liver from Gestational to Adult Development. Activity-based protein profiling was applied to the entire complement of samples characterized by global proteome measurements. Using the AMT tag approach for relative quantification, 24 P450 enzymes were identified throughout the array of samples by ABPP (**Figure 4**). These proteins were grouped by activity patterns of LC-MS identification and loosely fall into the previously described P450 ontogeny classifications and include class 1 (those with a significant decrease in postnatal samples) 3A7, 4A11, 4F12, 19A1, 51A1; class 2 (consistent from gestation through adulthood) 3A4, 4F2, 4F3, 7B1, 8B1, 20A1; and class 3 (significant increase in postnatal samples) 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 2J2, 3A5, 4F11, 4V2, 27A1.

Public Deposition of Data. The mass spectrometry proteomics data has been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD002781 and 10.6019/PXD0027.

Correlation of P450 Gene and Protein Expression and Enzyme Activity. P450 1-3 family enzymes play major roles in drug metabolism, while P450 family 4 enzymes primarily metabolize endogenous compounds such as eicosanoids. Prior reports on the P450 1A subfamily have been contentious arguing for little to no expression, or for highly variable expression across prenatal development (Omiecinski et al., 1990; Murray et al., 1992; Cazeneuve et al., 1994; Hakkola et al., 1994; Shimada et al., 1996; Hakkola et al., 1997; Sonnier and Cresteil, 1998; Choudhary et al., 2005; Bieche et al., 2007). By qRT-PCR,

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we determined gene expression levels are negligible to very low, but some extreme outliers were identified (e.g., *CYP1A2* in liver 633; **Figure 2**). Global proteomic analysis showed variable levels of P450 1A2 expression for all prenatal samples at several fold less than postnatal samples, and detected active 1A2 proteoforms in a single prenatal sample. Active enzyme profiles only resulted in the identification of 1A2 in a single prenatal sample. Our qRT-PCR results also indicate minimal *CYP1A1* expression and moderate *CYP1B1* expression, yet no measurement of protein abundance was made by proteomics for either P450 1A1 or 1B1. In a prior qRT-PCR analysis, only 3 of 66 samples that were older than 22 weeks were found to express *CYP1B1* (Hakkola et al., 1994; Hakkola et al., 1997; Bieche et al., 2007).

All P450 2A members displayed the general trend of increasing both abundance and activity with increasing age. The P450 2C subfamily is responsible for the metabolism of ~30% of all clinical drugs in adults. The application of several therapeutics used for pediatric ailments are metabolized by 2C9 and 2C19, an association between 2C9 and sudden infant death syndrome (Treluyer et al., 2000), and a lack of prior 2C9 versus 2C19 ontogeny profiling (Treluyer et al., 1997; Koukouritaki et al., 2004), led us to focus on these two 2C enzymes. Consistent with previous reports (Hines, 2008), our qRT-PCR analyses reveals elevated *CYP2C19* and minimal *CYP2C9* expression. We identified P450 2C9 in two prenatal samples and 2C19 in one prenatal sample via global proteomics, yet no active proteoforms were detected by ABPP. This may be due to our cohort being primarily from early to mid-second trimester and/or a difference in sensitivity comparing prior antibody-based approaches to LC-MS based ABPP.

Though minimally expressed in the adult, P450 2D6 accounts for the oxidative metabolism of ~12% of all clinical drugs (Shimada et al., 1994). Previous studies have not found that prenatal *CYP2D6* mRNA levels correlated well with immunochemical protein assays or metabolic activity (Treluyer et al., 1991). In our study, minimal protein

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expression for 2D6 was measured in ~50% of prenatal samples and active 2D6 was only measured in three second trimester prenatal samples. Poor correlation between transcription, translation and activity in prenatal samples has led to suggestions of a translational control mechanism that may overlay any transcriptional regulation (Hines, 2008).

Aside from the minor role played in xenobiotic metabolism, P450 2J2 converts arachidonic acid to biologically active eicosanoids, which may play integral roles in development (Lee et al., 2010). Expression of *CYP2J2* was low but detectable in ~20% of prenatal samples, but proteomic analysis positively detected P450 2J2 throughout development, but at several fold higher in neonates and infants. ABPP profiles for 2J2 mirrored the global proteomic results but with identification made for only ~70% of all samples.

Nearly half of all clinical drugs are oxidatively metabolized by the P450 3A subfamily (Hines, 2008). This subfamily dominated our measurements for gene and protein expression and ABPP-determined activity, consistent with many prior reports (Lacroix et al., 1997; de Wildt et al., 1999; Stevens et al., 2003; Choudhary et al., 2005; Bieche et al., 2007). P450 3A7 is a class 1 enzyme with high expression during prenatal development and rapidly waning after birth (Lacroix et al., 1997; Stevens et al., 2003; Hines, 2007; Hines, 2013). In our panel, *CYP3A7* is the most highly expressed gene in ~80% of samples. Protein and activity profiles showed high levels of 3A7 in all prenatal samples and a swift decrease in both measurements after birth. P450 3A4, the major adult isoform, had relatively high gene expression, protein and activity in the panel, but with significant increase in all postnatal measurements (class 3).

The P450 4 family encodes constitutive and inducible isozymes including the P450 4A subfamily, a hormonally regulated group that is induced by peroxisome proliferating chemicals (Simpson, 1997). In all life stages, we detected consistent gene

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and protein expression for the fatty acid oxidizing 4A11, though activity levels significantly decreased postnatally. The P450 4F subfamily is recognized for its dual role in modulating the concentrations of eicosanoids during inflammation as well as in the metabolism of clinically significant drugs (Kalsotra and Strobel, 2006). We did not perform qRT-PCR on this subfamily, but all members identified in our proteomic analysis (4F2, 4F3, 4F11, 4F12, 4F22) were either missing from most prenatal samples (4F11, 4F22), or were measured but had significantly higher levels in postnatal samples. Interestingly, a subset of prenatal samples had moderate levels of 4F12 activity, yet no activity was measured in postnatal samples. 4V2 protein expression was detected in many samples with abundance increasing with age, yet activity was only measured in one prenatal sample and ~30% of postnatal samples.

Cytochrome P450s from non CYP1-4 families primary role tends to involve endogenous metabolism and steroid production, processes critical for prenatal development and viability. P450 7B1 catalyzes the 7 α -hydroxylation of several steroid substrates, including those involved in hormonal signaling (Tang et al., 2006). Prenatal ontogeny studies have revealed 7B1 to be primarily extrahepatic, with little or no liver expression (Tang et al., 2006). Our qRT-PCR measurements identified detectable levels in only 20% of the samples, consistent with prior analyses. Protein expression levels were measured for all but one prenatal sample but these levels decreased with age and activity was nonexistent for all but two prenatal samples.

In contrast, P450 8B1, a sterol 12 α -hydroxylase that catalyzes cholic acid synthesis and controls soluble cholesterol levels (Gafvels et al., 1999), is highly expressed in nearly all prenatal livers examined in our study and protein was detected in every sample with a significant increase in postnatal expression. Active P450 8B1 was also detected in most samples, yet there was no significant change in activity between age groups.

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P450 19A1 converts androgens to estrogens, and our analysis shows that the gene for this enzyme was expressed in ~50% of second trimester prenatal, yet proteomic analysis identified 19A1 in all but one prenatal sample and activity in ~40% of the prenatal samples. No 19A1 identification was made in postnatal samples.

The “orphan” enzyme, P450 20A1, has no known function. Our analysis measured low levels of gene expression in both trimesters. Proteome analysis identified similar levels of 20A1 in all but one sample across all life stages, and activity was measured for this enzyme in ~40% of samples.

Finally, P450 51A1 stands out because it was identified in the whole sample panel save one juvenile sample and activity was measured in ~80% of samples. Both measurement profiles mirrored each other in that levels significantly decreased after birth. We note that some samples showed no activity at all: FL_853, FL_880, AL_057, and AL_551. These samples were not removed from analyses because protein expression was identified by global proteomics in the samples. However, it is likely that some aspect of the processing of these samples altered the functional capacity of enzymes.

DISCUSSION

Evaluation of gene and protein expression is important to understanding the developing liver, but does not provide insight into actual functional metabolic capacity. Enzyme activity must be measured to truly understand metabolic potential. Traditional assays for P450 activity have limitations, such as interpretation of activity data being complicated by the overlapping substrate specificities of P450 enzyme subfamilies. Given the considerable number of liver P450 enzymes, and the limitation of sample resources (particularly with gestational samples), it is intractable to run a large enough suite of traditional assays to evaluate activity of a broad swath of P450s. Activity-based

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protein profiling (ABPP) can overcome these challenges through the use of chemical probes that irreversibly label only functionally active P450 enzymes which can then be isolated. Coupled to LC-MS analyses, relative quantitation of P450 activities can be measured. We have employed a multi-omic analysis to provide a comprehensive comparison of life-stage P450 ontogeny at the gene expression, protein expression, and protein activity levels. **Figures 2, 3, and 4** reveal enzyme activity trends that do not always correlate with protein or gene expression, illuminating the importance of ABPP to profile the ontogeny of activity to get a more accurate measure of the functional capacity of individual P450s in each liver sample.

As discussed throughout, we have characterized P450 enzyme ontogeny on a class basis as delineated by Hines (Hines, 2013). In evaluating our ABPP data on a class basis and comparing to prior results we identified P450 3A7 as the dominant active class 1 isoform; however, we also newly classified four P450s (4A11, 4F12, 19A1, and 51A1) into class 1. We also used ABPP to newly classify five class 2 P450s (4F2, 4F3, 7B1, 8B1, and 20A1) and six class 3 P450s (2A6, 2C8, 2J2, 4F11, 4V2, and 27A1). Of those P450s classified by Hines the vast majority correlate with our data including 1A2, 2C9, 2D6, 2E1, and 3A7. Interestingly, P450s 2B6, 2C19, and 3A5 were previously characterized as class 2 enzymes, but in both our global proteomics and ABPP analyses we observe these as occurring primarily with only postnatal expression. Importantly, new classifications point to the strength of ABPP because activity assays are not available for many individual P450 enzymes, and/or no prior transcriptome or RT-PCR ontogeny studies have been performed which identified these P450s.

Cytochrome P450 protein expression is typically quantified by antibody-based western blotting, and activity by substrate-based assays that can be nonselective for single P450 isoforms. An important feature of our global proteome and ABPP measurements is the ability to rapidly yield relative quantification of P450 abundance

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and activity of numerous individual P450 enzymes (Wright and Cravatt, 2007; Wright et al., 2009). **Figure 5** (see also *Supplemental Figure 1*) displays the correlation of age-dependent protein expression and activity for P450s measured by both global and ABPP proteomics (note, P450s 1A1, 2W1, 4F22, 17A1, and 39A1 were identified only in global proteome analyses; see *Supplemental Datasets 8 and 9*). What is quickly apparent is that there is rarely a direct correlation between P450 activity and protein expression; within **Figure 5** some interesting age-dependent features can be readily identified. First, 3A7 aside, the key drug metabolizing families are far more abundant and active after birth, and both features are somewhat correlated for most family 1-3 enzymes. Our postnatal sample analyses show quite a different trend in which activity is considerably higher than abundance. This indicates highly active metabolic capacity for xenobiotics, and demonstrates that quantifying protein abundance alone inadequately illustrates P450 metabolic capacity. Additionally, with regard to therapeutic drug dosing, it is clear that developmental stages are greatly altered in the activity of P450 enzymes involved in drug metabolism. During infancy and juvenile stages, P450 activities are generally lower than expression. This suggests that drug dosing must be carefully considered for pediatric therapies.

In summation, profound changes occur in P450 gene and protein expression and activity throughout human development. Life-stage-dependent differences in P450 activity likely play a pivotal role in not only the disposition and efficacy of therapeutic drugs, but also in the metabolism of other xenobiotics and environmental agents. These results combined with prior findings can lead to more judicious predictions of drug metabolism that will result in safer dosing. We do note that ABPP is not absent of potential issues that should be carefully evaluated, such as the method as performed relies on the native expression and activity of the requisite NADPH-dependent P450 reductase within each sample, and the method relies on each individual P450's catalytic

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activity toward the activity-based probes. In light of these potential issues, it is most appropriate to compare the probe-determined activity of a single P450 across samples, as opposed to direct comparison of individual P450 activities since the probes do not specifically measure a catalytic rate. Differences when correlating global proteomic abundances to ABPP measurements may be due to differences in expression of electron transferring accessory enzymes such as NADPH-dependent P450 reductase (POR) or cytochrome b5. Differing POR:P450 ratios can alter activities, as can protein-protein interactions that result in P450 inhibition or cooperativity (Denisov et al., 2009; Reed and Backes, 2012). In the near future we anticipate that ABPP will be applied to broader cohorts of samples to expand our findings, and enhance our understanding of P450-mediated oxidative metabolism throughout human life stages.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Sadler, Smith, Corley, Wright

Conducted experiments: Sadler, Nandhikonda,

Performed data analysis: Sadler, Nandhikonda, Webb-Robertson, Ansong, Anderson,
Wright

Wrote or contributed to the writing of the manuscript: Sadler, Nandhikonda, Ansong,
Corley, Wright

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FOOTNOTES

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- b) Reprint requests should be sent to:

Aaron T. Wright
902 Battelle Blvd
Box 999, MSIN J4-02
Richland, WA 99352
Aaron.Wright@pnnl.gov

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FIGURE LEGENDS

Figure 1. (A) Structure of P450 activity-based probes (P450-ABPs). (B) One alkyne (red) is oxidized by a functionally active P450 enzyme in a mechanism-dependent manner yielding a reactive ketene that irreversibly reacts with and inactivates the now probe-labeled P450. The second alkyne enables click chemistry-mediated addition of a biotin group for subsequent streptavidin resin-based enrichment and quantitative LC-MS analysis of probe labeled P450s.

Figure 2. qRT-PCR of P450 genes involved in drug metabolism and steroidogenesis in prenatal livers. Relative gene expression levels of human P450 genes in prenatal livers were determined by qRT-PCR. For each gene, the average threshold cycle (Ct) was determined from the mean of three technical reactions \pm SD and normalized against a housekeeping gene (GAPDH). Relative expression values are given as fold-change against the internal reference, GAPDH. Samples below the asterisked sample are all from TM1, all other samples are TM2. The specific values corresponding to the heatmap are provided in *Supplemental Dataset 4*; unprocessed raw values for the replicates are in *Supplemental Dataset 5*.

Figure 3. Global P450 protein expression, quantified by the AMT tag approach. Comparison of the average expression of the prenatal first trimester (TM1) and prenatal second trimester (TM2) groups to the average expression of neonate/infant, juvenile and adult groups is demonstrated through a q-test value, which represents a significance metric applied to the various statistical analyses. q-test values < 0.05 represent high confidence determination that protein abundance values increase or decrease when going from prenatal to postnatal samples. Samples increase in age from left to right.

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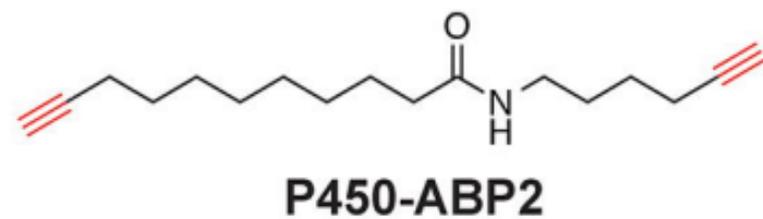
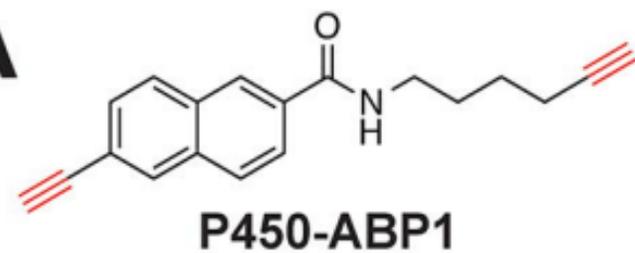
Values correlating to the heatmap are provided in the *Supporting Dataset 7*. Quantitative LC-MS values at the peptide level are provided in *Supplemental Dataset 8*.

Figure 4. Activity-based protein profiling of P450 enzymes, quantified by the AMT tag approach. Comparison of the average probe enriched P450s from the prenatal first trimester (TM1) and prenatal second trimester (TM2) groups to the average expression of neonate/infant, juvenile and adult groups is demonstrated through a q-test value, which represents a significance metric applied to the various statistical analyses. q-test values < 0.05 represent high confidence determination that protein abundance values increase or decrease when going from prenatal to postnatal samples. Samples increase in age from left to right. Values correlating to the heatmap are provided in *Supporting Dataset 9*. Quantitative LC-MS values at the peptide level are provided in *Supporting Dataset 10*.

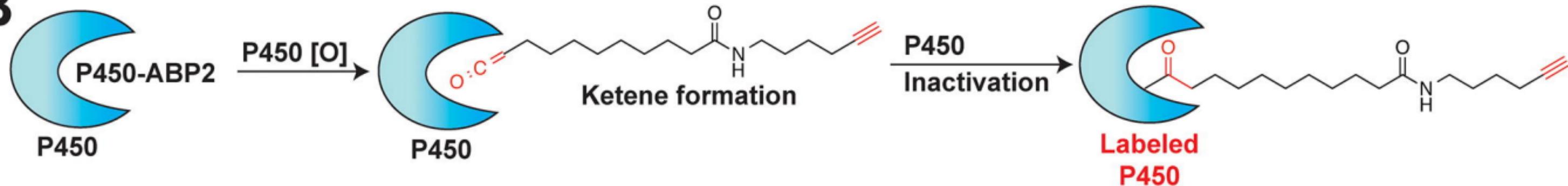
Figure 5. Comparison of P450 protein abundance to P450 activity (as determined by ABPP). Activity and protein expression values are averaged by life stage, and normalized on a 0-1 scale for each individual P450. Thereby, points of highest expression or activity for each P450 are observed as increasingly red. See also *Supplementary Figure 1* in the *Supporting Information*.

Figure 1

A



B



Labeled
P450

1. Click Chemistry
2. Enrichment
3. MS Analysis

Quantitative characterization
of functionally active P450s

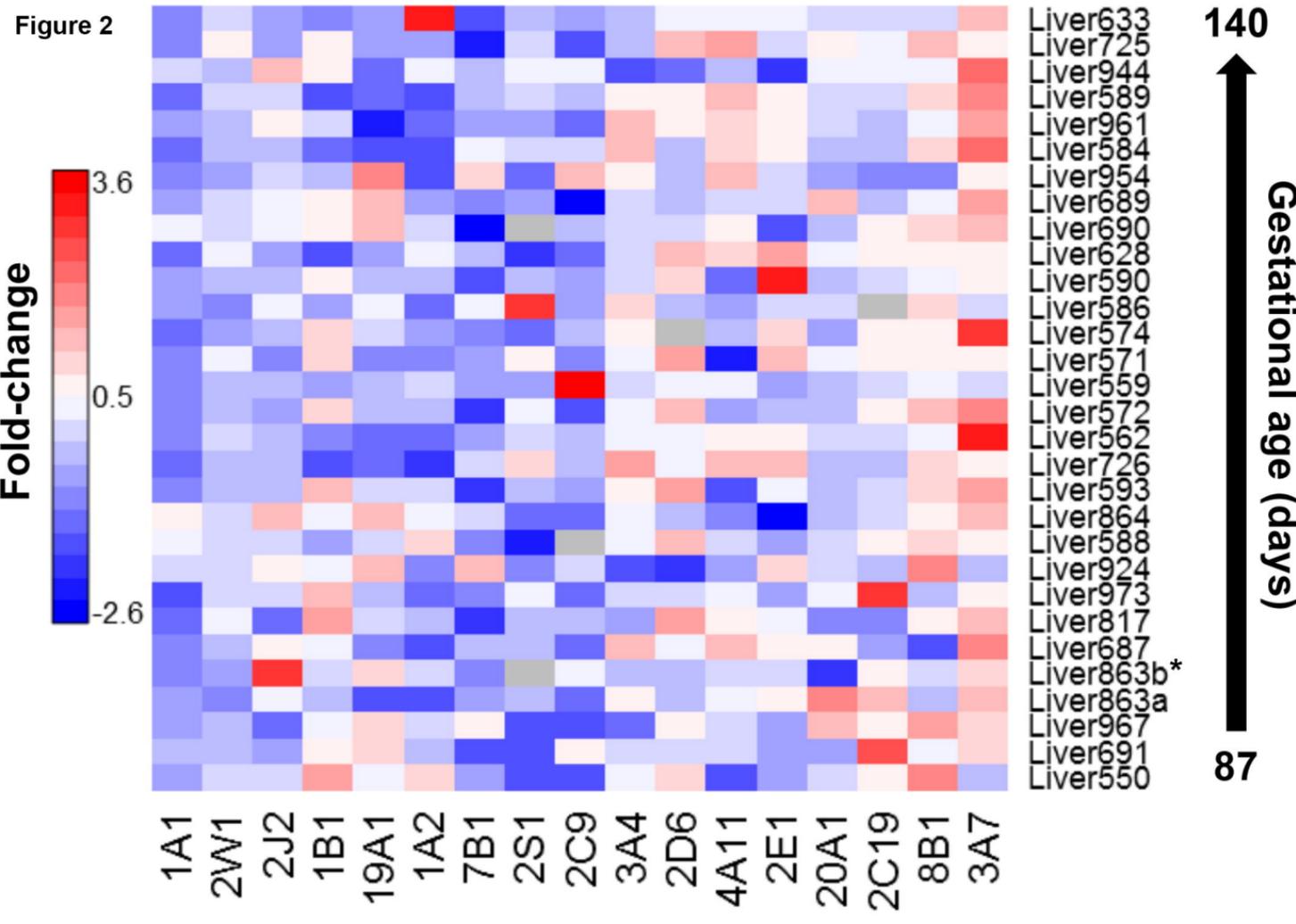


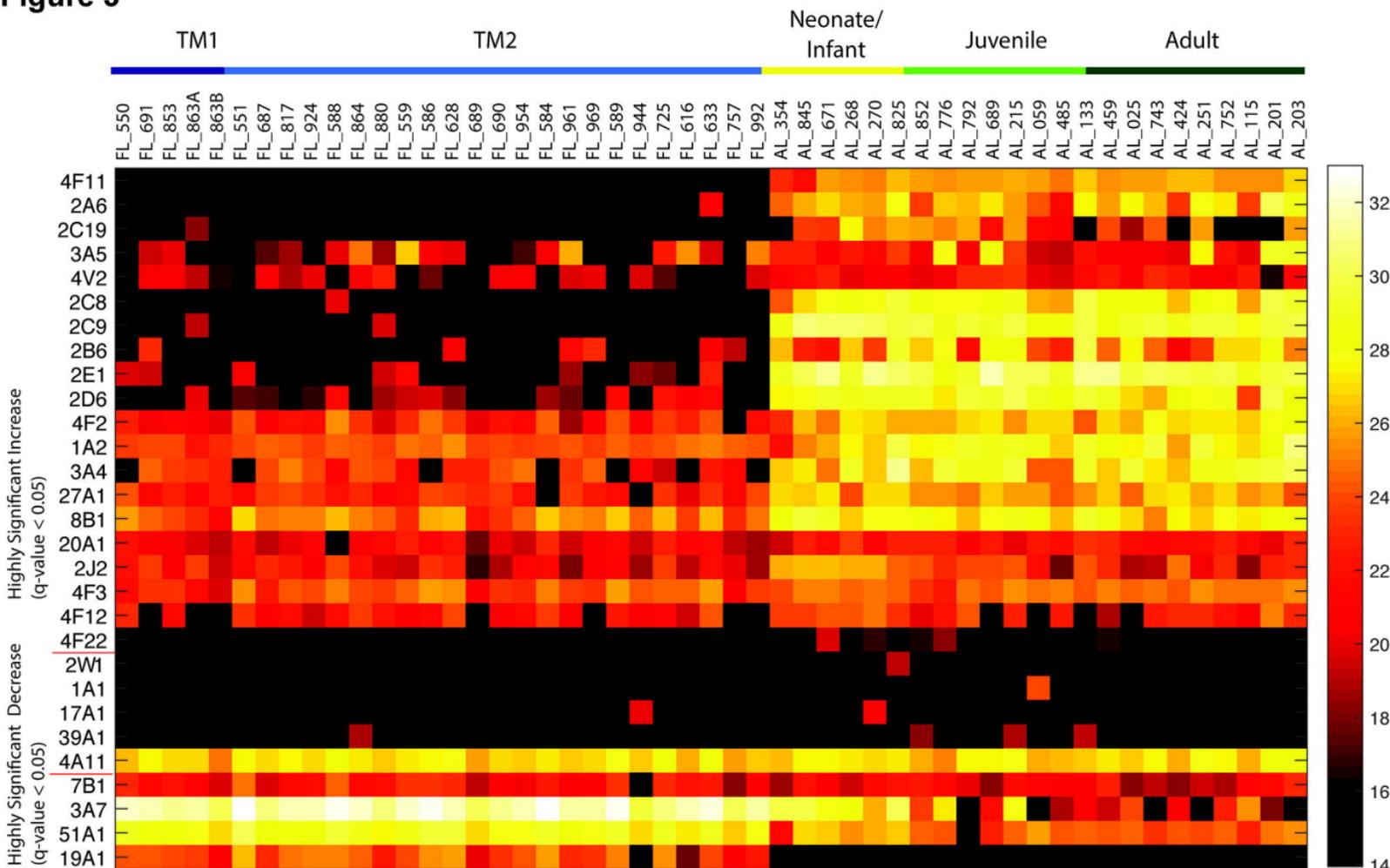
Figure 3

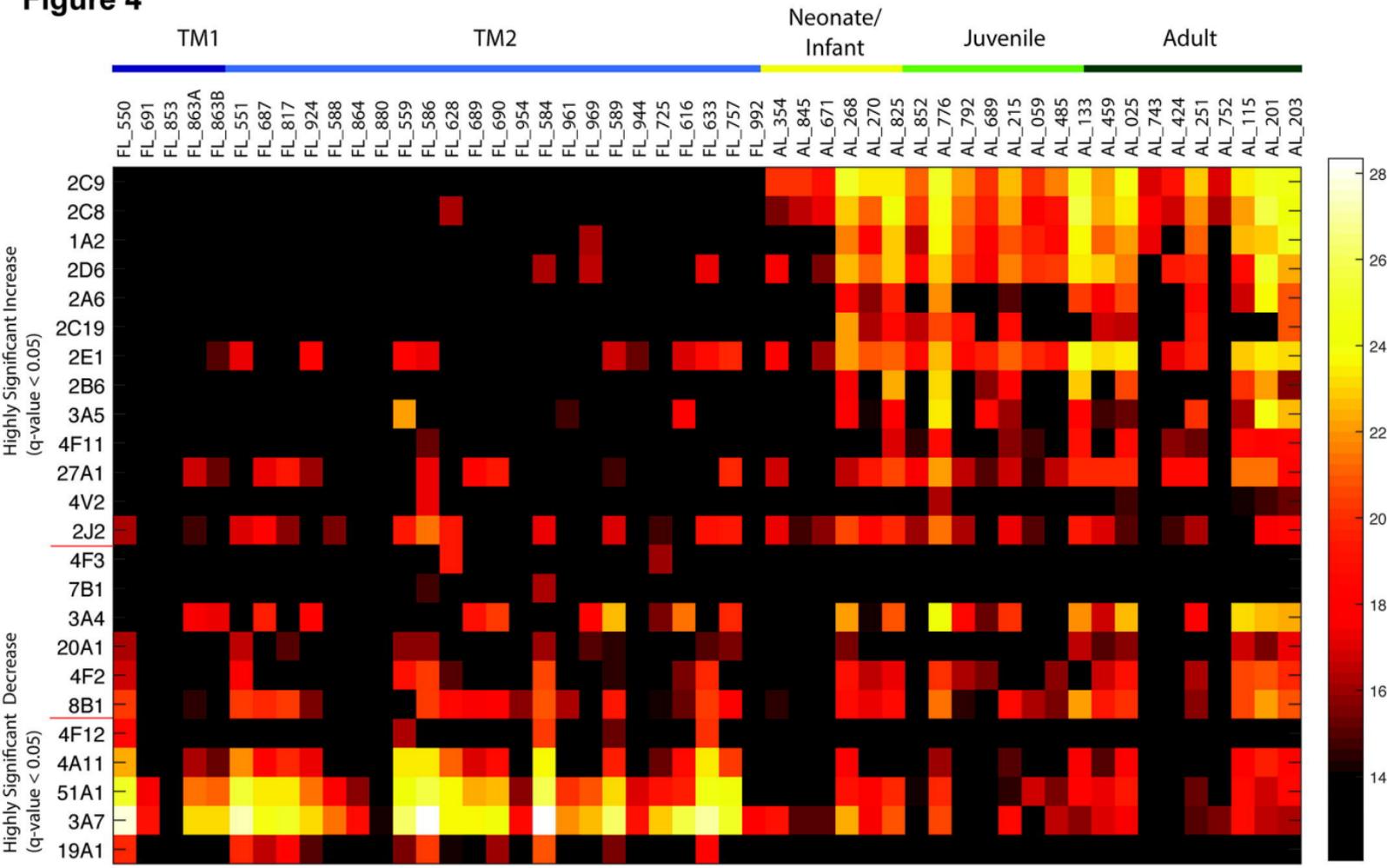
Figure 4

Figure 5