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Regulation of CYP3A5 in Pancreatic Ductal Adenocarcinoma

Abstract

CYP3A5 is a drug metabolism enzyme that is polymorphically expressed in normal tissues and expressed in high amounts in some cancers, including pancreatic ductal adenocarcinoma (PDAC). There have been several published studies regarding CYP3A5 regulators, but there is still much to be discovered concerning CYP3A5 regulation in cancer. To further understand the regulation of CYP3A5, a high-throughput screen was performed in the AsPC-1 PDAC cell line. This screen employed small interfering RNA (siRNA) targeting transcription factors and chromatin-associated proteins. We identified siRNA that decreased CYP3A5 mRNA and protein levels in AsPC-1 cells and probed into the potential mechanisms. Taken together, this study brought to light a previously undescribed regulator of CYP3A5 expression.

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In Partial Fulfillment
Of the Requirements for the Degree
Master of Science
From The University of Tennessee

By
Jessica D. Hoyer
June 2019

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DEDICATION

This work is dedicated to my grandfathers, Dr. D. Pat Maddox and Charlie R. McCain.

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I would like to thank Dr. Taosheng Chen for bringing me into his lab where I could learn and grow as a scientist and be a part of the St. Jude research community. Also, thank you to Drs. Meiyun Fan, Kevin Freeman, Mark Hatley, and Susan Senogles for serving on my committee and supporting me throughout graduate school. I must thank Drs. Jesse Bakke, Jordan Beard, Milu Cherian, Alexa Farmer, Yueming Wang, Charlie Wright, and all members of the Chen lab for their guidance, friendship, instruction, and for answering my many questions. Special thanks and love go to Jing Wu for being my constant friend and encourager.

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ABSTRACT

CYP3A5 is a drug metabolism enzyme that is polymorphically expressed in normal tissues and expressed in high amounts in some cancers, including pancreatic ductal adenocarcinoma (PDAC). There have been several published studies regarding CYP3A5 regulators, but there is still much to be discovered concerning CYP3A5 regulation in cancer. To further understand the regulation of CYP3A5, a high-throughput screen was performed in the AsPC-1 PDAC cell line. This screen employed small interfering RNA (siRNA) targeting transcription factors and chromatin-associated proteins. We identified siRNA that decreased CYP3A5 mRNA and protein levels in AsPC-1 cells and probed into the potential mechanisms. Taken together, this study brought to light a previously undescribed regulator of CYP3A5 expression.

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LIST OF ABBREVIATIONS

ACC	Adrenal Cortical Carcinoma
BPA	Branch Point Adenosine
CSC	Cancer Stem Cell
CYP	Cytochrome P450
DNBP	DNA-RNA-Binding Protein
HCC	Hepatocellular Carcinoma
KD	Knock Down
KO	Knock Out
NT	Non-Targeting
PAF1C	Polymerase-Associated Factor 1 Complex
PDAC	Pancreatic Ductal Adenocarcinoma
RT	Room Temperature
siRNA	Small Interfering RNA
SNP	Single Nucleotide Polymorphism
snRNA	Small Nuclear RNA
snRNP	Small Nuclear Ribonucleoprotein
SSMD	Strictly Standardized Mean Difference

CHAPTER 1. INTRODUCTION

Drug Metabolism and the Cytochrome P450s

Drugs and environmental chemicals (xenobiotics) as well as endogenous compounds (endobiotics) must go through biotransformation and inactivation in the liver before exiting the body. Drug metabolism is generally broken down into two phases. Phase I occurs when polar groups are attached to hydrophobic substrates via enzymes that catalyze hydrolysis, reduction, and oxidation reactions. Phase II occurs when enzymes, including UDP-glucuronosyltransferases and sulfotransferases, mediate conjugation reactions where groups such as glucuronate, sulfate, glutathione, and amino acids are conjugated to the drug (Iyanagi, 2007). These solubilized drug metabolites are then eliminated from the body as less harmful waste products.

The human cytochrome P450 (CYP) family of hemoproteins are Phase I enzymes. They are classified as monooxygenases which metabolize small molecule drugs and other xenobiotics by oxidation. This oxidation results in the activation of prodrugs or the inactivation and eventual clearance of other compounds. The CYP family consists of nearly 60 members stratified into 18 families based on sequence homology (Nelson, 2009; Nebert, 2013).

The CYP3A Family

One important subfamily of CYPs, the CYP3A subfamily, is responsible for approximately 50% of clinical drug detoxification in the liver (Zanger, 2013). The *CYP3A* gene cluster is located on chromosome 7q22.1 and consists of 4 genes: *CYP3A4*, *CYP3A5*, *CYP3A7*, and *CYP3A43*, and two pseudogenes: *CYP3AP1* and *CYP3AP2*. *CYP3A4* is the most well-studied gene as it is highly expressed in the liver, with high individual variability (>100-fold). The other three subfamily members, including *CYP3A5*, are also expressed in the liver, but at lower levels than *CYP3A4* (Zanger, 2013). A mass-spectrometric quantification of the CYP content of donor human liver microsomes showed that of the total CYP3A protein measured, *CYP3A4* made up 85.4% and *CYP3A5* made up 5.4% (Ohtsuki, 2012). *CYP3A5* is expressed at low levels in the liver and in extra-hepatic tissues such as the gastrointestinal tract, kidney, prostate, adrenal gland, and lung (Raunio, 2005; Bolbrinker, 2012; Koch, 2002).

Not much is known about the regulation of *CYP3A5*, however, *CYP3A5* has been shown to be transcriptionally regulated by PXR, CAR, GR, VDR, and HNF4A (Burk, 2004; Maguire, 2012; Basseville, 2011; Hukkanen, 2003; Jover, 2001). One study reviews the contrasting data about the inducibility of *CYP3A5* and goes on to clearly demonstrate how *CYP3A5* is in fact inducible in hepatocytes via PXR and CAR in response to drugs (Burk, 2004).

Additionally, there is documentation of elevated CYP3A5 in certain cancer types, such as adrenal cortical carcinoma (ACC), pancreatic cancer, colorectal cancer, and ovarian cancer (Noll, 2016; Downie, 2005; Kumarakulasingham, 2005; Ma, 2018). With this drug metabolism-associated gene being upregulated in cancer, it brings one to ask what function it performs in cancer and by what mechanism.

Polymorphic CYP3A5

To address CYP3A5 functioning, one must consider the fact that CYP3A5 is highly polymorphic. The wild type *CYP3A5* allele, also called *CYP3A5*1*, contains 13 exons that are translated into a 502-amino acid protein (**Figure 1-1**). Single nucleotide polymorphisms (SNPs) determine the genotype of *CYP3A5*, which determines expression status, and therefore the activity of the enzyme. Four alleles exist for *CYP3A5*: *CYP3A5*1*, *CYP3A5*3*, *CYP3A5*6*, and *CYP3A5*7*. *CYP3A5*1* is the active, full length transcript. *CYP3A5*3* contains a SNP entitled rs776746, or 6986A>G, that dictates activity. *CYP3A5*1* contains an A in this position, where a G results in the inactive form *CYP3A5*3*. *CYP3A5*3* renders the protein inactive by revealing a cryptic splice site that leads to a premature stop resulting in a truncated protein. *CYP3A5*6* and *CYP3A5*7* are two null alleles that are less commonly found.

Many reports display the racial disparity of expression of active CYP3A5. Less than 10% of Caucasians express active *CYP3A5*1*, while greater than 60% of African populations express *CYP3A5*1* (Zanger, 2013). In the normal liver CYP3A5 should be largely dispensable since CYP3A4 can compensate for loss of CYP3A5 activity, as CYP3A4 is the most prominent family member. An area in which this polymorphic expression is of concern is drug administration regarding toxicity and response. Though some propose CYP3A5 only plays a minor role in drug metabolism (Westlind-Johnsson, 2003), there is evidence that for some drugs, CYP3A5 is more important (Huang, 2004). The dose administered and potential toxicity of the immunosuppressive drug tacrolimus is associated with the genotype of CYP3A5. The intrinsic clearance of this drug is about 2-fold higher for CYP3A5 than CYP3A4. Individuals who are *CYP3A5*1* expressers show lower trough blood concentrations of tacrolimus, indicating that CYP3A5 plays a leading role in its clearance (Dai, 2006). Doses must be clearly monitored to avoid life-threatening nephrotoxicity that can occur if tacrolimus accumulates due to differences in drug clearance. This principle could potentially affect cancer treatment as well. These differences in functionality between these two main genotypes of *CYP3A5* could potentially have an impact on treatment options.

A recent paper demonstrates that CYP3A5 mediates basal and acquired drug resistance in a patient-derived xenograft (PDX) pancreatic ductal adenocarcinoma (PDAC) cell model (Noll, 2016). When treated with two tyrosine kinase inhibitors, knock down (KD) of CYP3A5 via siRNA rendered the cells more sensitive to drug treatment. Regarding regulation, this study demonstrates that basal expression of *CYP3A5* is mediated by HNF4A and drug-induced expression is mediated by PXR. This transcriptional relationship between *CYP3A5* and nuclear receptors is supported by

Figure 1-1. Splice Variants of CYP3A5

Schematic drawing of the gene and the cDNA structures of the major *CYP3A5* alleles. Exons are numbered and boxed. Exon 3B derived from an intronic sequence is labeled in red.

previous studies (Burk, 2004). Another interesting point is that PXR levels are correlated with total CYP3A5 levels, but the correlation coefficients rise when you isolate CYP3A5*1 expression. Targeting the HNF4A and/or PXR CYP3A5-regulatory pathways could be one therapeutic option to increasing drug sensitivity in CYP3A5-expressing tumors. Since *CYP3A5* is upregulated in several cancer types, including hepatocellular carcinoma (HCC), gastric carcinoma, and ACC (Noll, 2016), mechanistic studies could reveal the factors responsible for this upregulation.

Splicing and PHF5A

The Spliceosome

The spliceosome is a large assembly of small nuclear RNAs (snRNAs) and proteins that come together to form complexes called small nuclear ribonucleoproteins (snRNPs) designated U1, U2, U4, U5, and U6. This molecular machine localizes to nuclear speckles in eukaryotic cells and is the site for pre-mRNA splicing. Through a cascade of events, the spliceosome is responsible for processing out introns and ligating exons into mature mRNA that will be translated by the ribosomes into protein. The full process is summarized in an elegant publication (Lodish, 2000). Of interest to this project is the step in which U2 binds the branch point adenosine (BPA) to initiate lariat formation. The U2 snRNP is made up of the SF3A and SF3B subcomplexes. One of the SF3B proteins is called PHF5A. Along with PHF5A, SF3B1 and SF3B3 contain residues that make up the binding site for splicing modulators such as Pladienolide B, Herboxidiene, the Sudemycins, and Spliceostatin A. These compounds inhibit formation of the spliceosome and have been used to target the spliceosome as a cancer treatment by inducing apoptosis in tumor cells (Kashyap, 2015; Martinez-Montiel, 2016).

PHF5A

PHF5A is a small 12 kD protein in the PHD-domain containing protein family. While the PHF5A transcript contains some variability between species, the protein sequence in all vertebrates tested is 100% identical (Oltra, 2003) and is ubiquitously expressed in all tissues (Falck, 2013). PHF5A has seemingly diverse functions. One published mechanism for PHF5A is its role as a transcriptional cofactor. One study proposes that the PHF5A protein localizes to the nucleus and binds the proximal promoter of *Connexin43* (*Cx43*), enhancing the response of *Cx43* to estrogen (Oltra, 2003). A recent paper identifies PHF5A as a DNA-RNA-binding protein (DRBP) (Conrad, 2016). This paper proposes that PHF5A is a DRBP that couples RNA splicing to transcription, since it binds to *Cx43* and acts as a bridge between splicing proteins and helicases (Rzymiski, 2008). Another name for PHF5A is SF3b14b, which was identified as a member of the SF3b complex, a part of the U2 snRNP of the spliceosome (Will, 2002). A recent review mentions several compounds reported to inhibit the spliceosome, several by targeting the SF3B1 subunit (Effenberger, 2017). Two of these inhibitors are

Pladienolide B and Herboxidiene. PHF5A also stabilizes the polymerase-associated factor 1 complex (PAF1C). The human PAF1C is a 6-membered protein complex responsible for transcriptional elongation and is made up of PAF1, CDC73, WDR61, LEO1, RTF1, and CTR9. Interestingly, PAF1 is a marker that is expressed on pancreatic cancer stem cells (CSCs), and may play a role in CSC self-renewal and drug resistance of these pancreatic CSCs (Vaz, 2014). PHF5A stabilizes the PAF1C and knocking down PHF5A results in disruption of the PAF1C and decreased transcriptional elongation of PAF1C target genes (Strikoudis, 2017).

Hypothesis and Specific Aims

We hypothesize that additional regulatory mechanisms exist for *CYP3A5*. Knowledge of these mechanisms could lend insight into therapeutic targets to overcome drug resistance, as well as adding to the body of knowledge of *CYP3A5* regulation. The goal of this study is two-fold: 1) to perform a transcription factor and chromatin-associated protein siRNA screen for potential regulators of *CYP3A5* in a PDAC cell model, 2) to follow up on top screen hits, such as PHF5A, and identify the mechanism of their regulation.

CHAPTER 2. MATERIALS AND METHODS

Cell Culture

The cell lines AsPC-1 (CRL-1682), HPAF-II (CRL-1197), PANC-1 (CRL-1469), MIA PaCa-2 (CRL-1420), SU.86.86 (CRL-1837), CAPAN-2 (HTB-80), CFPAC-1 (CRL-1918), Panc02.13 (CRL-2554), HCT 116 (CRL-247), HEPG2-C3A (CRL-10741), and LS180 (CRL-187) were obtained from the American Type Culture Collection (ATCC; Manassas, VA). SNU-C4 cells were obtained from KCLB (Cat# 0000C4, KCLB). Huh-7 cells were a gift from a collaborator from Japan. Cells were cultured in media per ATCC website media suggestions with FBS (HyClone; Logan, UT) and 1% Penicillin-Streptomycin (Life Technologies). Regarding passaging, cells were washed with 1X PBS (Gibco), trypsinized for 3-5 min, and neutralized with cell-type specific media. Cells were counted and 2-3 mil cells, depending on the cell type, were passaged into a new T-75 flask. Plating media for immunofluorescence experiments was RPMI-1640 without phenol red or antibiotics and supplemented with 10% FBS. Cells were cultured and maintained in a humidified incubator at 5% CO₂ at 37 °C.

siRNA

SMARTpool siGENOME siRNA sets were used to target the following transcripts (Dharmacon): CYP3A5 (M-009684-01-0005), NR1I2 (M-003415-02-0010), HNF4A (M-003406-02-0005), PHF5A (M-014987-01-0005), SMARCD1 (M-017244-01-0005), ZNF354A (M-007685-01-0005), BARX2 (M-012395-01-0005), CDX2 (M-015636-01-0005), GTF3C1 (M-012581-01-0005), ZNF397 (M-014870-00-0005), ZFH3 (M-015412-01-0005), KDM5A (M-003297-03-0005), PPARG (M-003436-02-0005), CREBBP (M-003477-02-0005), GATA3 (M-003781-01-0005), HIF1A (M-004018-05-0005), MED17 (M-006312-02-0005), RNF112 (M-006588-01-0005), KLF13 (M-006994-01-0005), SLC4A10 (M-007580-01-0005), TFAP4 (M-009504-00-0005), CDY2A (M-010346-01-0005), NOTCH2 (M-012235-00-0005), SF3B1 (M-020061-02-0005), SF3B3 (M-020085-01-0005), PAF1 (M-020349-01-0005), WDR61 (M-014614-00-0005), CDC73 (M-015184-00-0005), CTR9 (M-032246-01-0005), LEO1 (M-016579-00-0005), and RTF1 (M-014104-01-0005). Individual siNT5 was used as a control siRNA in all experiments (Dharmacon). For the top 5 hit siRNA deconvolution, the following siGENOME sets of 4 were used (Dharmacon): PHF5A (MQ-014987-01-0002), SMARCD1 (MQ-017244-01-0002), ZNF354A (MQ-007685-01-0002), BARX2 (MQ-012395-01-0002), and CDX2 (MQ-015636-01-0002).

siRNA Transfection

For qPCR and western blotting readout experiments, cells were counted in duplicate using the Cellometer Auto T4 (Nexcelom Bioscience). Per well, 5x10⁵ cells in 2 ml media, media according to cell type, were seeded in 6-well plates (Corning Costar)

for reverse transfection using the RNAiMAX transfection protocol (Invitrogen). For a 6-well plate, 2 ml was the total volume per well: 400 μ l transfection solution and 1600 μ l growth media. The transfection solution for one well consisted of the following: 400 μ l Opti-Mem, 6 μ l RNAiMAX, and 250 nM siRNA. First, 6 μ l RNAiMAX was diluted in 200 μ l Opti-MEM and mixed well. Second, 5 μ l siRNA from 10 μ M stock was diluted in 200 μ l Opti-MEM and mixed (ex: 5 μ l of a 10 μ M stock siRNA solution goes into 400 μ l transfection solution). Third, the two tubes were combined, mixed well, and incubated for 10-15 min at room temperature. siRNA was used at a final concentration of 25 nM. 2 ml media per well was added 24 hr post-transfection. 72 hr post transfection, media and cells were collected and downstream RNA isolation and qPCR was performed. When double KD was employed, and equal amount of siNT was supplemented in control wells to equalize the total amount of siRNA in each well.

For immunofluorescence optimization experiments, reverse transfection was also used. The cell suspension was pipetted through a cell strainer to achieve single cells, then 25 μ l media containing 1.5×10^4 cells was seeded per well in 96-well plates (Cell Carrier), overlaying siRNA with a final concentration of 25 nM. Each well contained 5 μ l 250 nM siRNA, 20 μ l OPTI-Mem, and 0.2 μ l RNAiMAX. 24 hr later, 50 μ l media was added. For lipid optimization, RNAiMAX (Invitrogen) and red and blue Dharmafect (Dharmacon) were tested. 72 hr post-transfection, cells were collected and downstream RNA isolation and qPCR was performed, see “RNA Isolation and qPCR” section below.

TaqMan qPCR Probes

For qPCR, 20X FAM-labeled TaqMan probes (ThermoFisher) were used to detect target gene expression. The TaqMan probes used were as follows: CYP3A5 (Hs01070905_m1), PHF5A (Hs00992149_g1), HNF4A (Hs00230853_m1), NR1I2 (Hs1114267_m1), SMARCD1 (Hs00161980_m1), ZNF354A (Hs01921591_s1), BARX2 (Hs00186346_m1), CDX2 (Hs01078080_m1), GTF3C1 (Hs00155360_m1), ZNF397 (Hs01061031_m1), ZFHX3 (Hs00199344_m1), KDM5A (Hs00231908_m1), PPARG (Hs01115513_m1), CREBBP (Hs00932878_m1), GATA3 (Hs00231122_m1), HIF1A (Hs00153153_m1), MED17 (Hs00188669_m1), RNF112 (Hs00993289_g1), KLF13 (Hs00429818_m1), SLC4A10 (Hs00222849_m1), TFAP4 (Hs01558245_m1), CDY2A (Hs00371558_s1), NOTCH2 (Hs01050702_m1), SF3B1 (Hs202782_m1), SF3B3 (Hs01035537_m1), PAF1 (Hs219496_m1), WDR61 (Hs228800_m1), CDC73 (Hs363810_m1), CTR9 (Hs00206060_m1), LEO1 (Hs292080_m1), RTF1 (Hs385532_m1), and GAPDH (Hs99999905_m1) as an endogenous gene expression control for target gene mRNA levels.

RNA Isolation and qPCR

Total RNA was extracted using the Maxwell 16 LEV simplyRNA Tissue Kit with the Maxwell 16 Research Instrument (Promega). Nucleic acid concentration was measured using the NanoDrop 8000 UV-Vis Spectrophotometer (ThermoFisher

Scientific). RNA was converted to cDNA by using the SuperScript VILO cDNA Synthesis Kit (Life Technologies). Target gene mRNA expression was detected by using 20X FAM-labeled TaqMan probes (ThermoFisher), Fast Advanced Master Mix (Life Technologies), and quantitated via the 7900HT Fast Real-Time PCR System (Applied Biosystems). The real-time PCR protocol found on page 14 of the TaqMan Fast Advanced Master Mix User guide, linked here https://tools.thermofisher.com/content/sfs/manuals/cms_084554.pdf, was followed exactly. Hold for 2 min at 50 °C, hold for 20 sec at 95 °C, and 40 cycles of 1 sec at 95 °C and 20 sec at 60 °C. 10.5 µl reactions (per well) were used as follows: 5 µl Fast Advanced Master Mix, 0.5 µl target gene TaqMan probe, 10 ng cDNA in a 5 µl H₂O/cDNA dilution. The fold-change in expression was calculated by using the comparative Ct ($\Delta\Delta C_t$) method, with the values of controls set to one (siNT5). All samples for every qPCR were tested in technical triplicate.

Western Blotting

Cells were lysed with Pierce RIPA buffer (ThermoFisher) plus Complete Mini protease inhibitor tablets (Roche) and incubated on ice for 1 hr, followed by sonication at 50% amplitude for a 5 sec pulse. Lysates were spun down at 16.2 xg for 5 min at 4 °C. Protein concentration was measured using the Pierce BCA Protein Assay kit (ThermoFisher). Protein samples were boiled in NuPAGE LDS 4X Sample Buffer (Life Technologies) for 5 min at 95 °C and subjected to gel electrophoresis using 4-12% Bis-Tris gels in 1X NuPAGE MES-SDS running buffer (Invitrogen). The resulting gels were transferred onto nitrocellulose membranes using the iBlot dry transfer system (Invitrogen). Membranes were blocked for 1 hr at room temperature (RT) with Odyssey blocking buffer PBS (LI-COR). The primary antibodies used were anti-cytochrome P450 3A5 antibody at 0.24 µg/ml (ab108624, ABCAM), anti-PHF5A at 0.24 µg/ml (15554-1-AP, Proteintech), anti-SF3B1 at 4 µg/ml (ab172634, ABCAM), and anti- β -actin 0.04 µg/ml (A5441, Sigma). Primary antibody was diluted in blocking buffer and membranes incubated overnight at 4 °C. The following morning membranes were incubated with species-specific infrared dye-conjugated secondary antibodies at a 1:10,000 dilution (LI-COR) for 1 hr at RT. 1X TBS-T was used to wash membranes 5 min 3X after incubation with both primary and secondary antibodies. Antibody-bound proteins were visualized using the Odyssey CLx (LI-COR). When necessary, membranes were stripped with 10 ml NewBlot Nitro Stripping Buffer 5X (LI-COR).

CYP3A5 Genotyping

Raw DNA was isolated via the High Pure PCR Template Preparation Kit protocol (Cat# 11796828001, Roche). To sequence the region of the *CYP3A5* gene where the rs776746 SNP is located, two rounds of PCR were performed and the resulting PCR product was sequenced. First, the *CYP3A5* gene (GenBank Accession #NG_000004.2) was amplified from the proximal promoter region to exon 7. Genomic DNA (300 ng) was amplified using 25 µl 2X Phusion High Fidelity PCR Master Mix with GC Buffer (New

England BioLabs, Cat# M0532S) with 1 μ l of 100 μ M each primer (Ztaq-pro-Ex7, forward and reverse) listed in **Table 2-1**. All genotyping primers were standard, desalted oligos (ThermoFisher Scientific). The first-round PCR: 2 min at 95 °C, 30 cycles of 10 sec at 98 °C, 10 sec at 55 °C, 4 min at 72 °C, and a 10 min final extension of 72 °C. Next, intron 3 was amplified by 2X Phusion with 1 μ l of 100 μ M Intron 3 primers (**Table 2-1**). Second-round PCR consisted of 5 min at 94 °C, followed by 30 cycles of 15 sec at 94 °C, 15 at 55 °C, 1 min at 72 °C, with a 10 min final extension of 72 °C. The PCR products were run out on a 2% agarose gel, cut out, and purified with the QIAquick Gel Extraction Kit (Qiagen, Cat# 28704). The resulting PCR product was Sanger sequenced using the second round Intron 3 primers to detect the identity of the SNP.

384-well Cell Number Determination

AsPC-1 cells were seeded in a CellCarrier-384 Ultra Microplate (Perkin Elmer) at concentrations of 6, 4, 2, and 1 thousand cells per well with 6 replicates of each cell dilution in 25 μ l plating media. Cell proliferation was monitored by using an IncuCyte ZOOM live-cell imaging system (Essen BioScience; Ann Arbor, MI). One image per well was captured every 3 hr for 72 hr. The percentage of confluent cells was calculated by using IncuCyte software.

Immunofluorescence Screen Conditions

For optimization experiments, 96-well plates were used. AsPC-1 cells (1.5×10^4 cells in 25 μ l media, per well) were seeded in 96-well plates (Cell Carrier), see “siRNA Transfection” section above for transfection details. For screening, 384-well plates were used to facilitate high-throughput, automated use. Briefly, an Aligent Biocel was used to perform an automated, high-throughput siRNA screen. An Aligent Bravo was used to stamp siRNA in assay plates, a Wellmate was used to dispense cells in media into the plates, and the plates were incubated at 37 °C in Liconic incubators. Each well contained 2.5 μ l 250 nM siRNA, 10 μ l Opti-MEM, and 0.1 μ l RNAiMAX. Cells were collected and pipetted through a cell strainer, counted in duplicate, and made up to a concentration so that there were 15 μ l cell solution per well with a total volume of 25 μ l in each well and 6,000 cells per well. The Dharmacon siTF library contained 6 plates, including 1 dummy plate. Controls were placed in columns 1, 2, 23, 24: 10 μ l 250 nm siRNA with 32 wells siNT5 and 32 wells siCYP3A5. Plate barcodes were scanned before use. After 24 hr, 25 μ l 10% FBS plating media was added to each well (Wellmate). Plate sets were fixed 48 and 72 hr post transfection. Set 3 was fixed at 48 hr, and sets 1 and 2 were fixed at 72 hr with paraformaldehyde at 4% final concentration for 20 min at RT. Cells were permeabilized with 0.25% final concentration Triton X-100 in PBS for 15 min. Cells were then blocked with Odyssey blocking buffer PBS for 1 hr at RT. The fixed cells were subjected to staining with anti-CYP3A5 antibody at 0.3 μ g/ml (ab108624, ABCAM) overnight at RT. The cells were then washed with cold 1X PBS-T and incubated with Alexa 647-labeled anti-rabbit secondary antibody (1:1000) (Invitrogen) at RT and DAPI at 5 μ g/ml for 1 hr. For whole-cell staining, the cells were subsequently incubated with

Table 2-1. Primer Sequences for *CYP3A5* Genotyping

Round	Primer Name	Forward Primer (5' to 3')	Reverse Primer (3' to 5')
1 st round	Ztaq-pro-Ex7	TATCTGTAATAATATGGATG GAACTG	CTTGTTCTAAACATAAG TTCTCTGTC
2 nd round	Intron 3	ATGGAGAGTGGCATAGGAGA TACC	CCATACCCCTAGTTGTA CGACACA

CytoPainter Phalloidin-iFluor 488 Reagent (1:4000) (Cat# ab176753, ABCAM) for 1 hr at RT. The cells were examined and analyzed by fluorescence microscopy using the InCell Analyzer (GE) and Dr. Yueming Wang performed batch analysis on the data.

Cell Proliferation Assay

Real-time cell growth in response to DMSO and splicing inhibitors was measured as the degree of cell confluence over time in culture plates. Confluence was determined using an IncuCyte HD live-cell imaging system (Essen BioScience). Cell densities for the growth curve determination were serially diluted 1:2 from 4 k to 0.5 k in 0.1% DMSO. Cells were plated in 96-well plates (Cell Carrier) leaving the edge wells with only plain media. Images were taken every 3 hr for 120 hr, 4 images per well. Cell proliferation curves were plotted using confluence values at specified time points for each treatment. For the cell growth studies upon splicing inhibitor addition, 4 k cells/well were plated in 96-well plates and 12 hr later, drugged with dilutions of drug, labeled time zero for the graphs. Immediately after drugging, 4 images per well were taken every 3 hr for 120 hr. These data were analyzed with the IncuCyte ZOOM software. Pladienolide B (CAS 445493-23-2) was purchased from Santa Cruz Cat# sc-391691, and Herboxidiene (CAS 142861-00-5) from Focus Biomolecules Cat# 10-1614-0200.

Inducible shRNA

A doxycycline-inducible PHF5A shRNA plasmid was constructed as previously described (Wiederschain, 2009; Wee, 2008)), using the pLKO-Tet-On parent plasmid (Tet-pLKO-puro, gift from Dmitri Wiederschain, Addgene plasmid #21915). Primers were designed using the nucleotide sequence of siPHF5A #2 (GCATATGTGATGAGTGTA); catalog number for Set of 4 siRNAs is MQ-014987-01-0002, siRNA #2 was the siRNA used as the basis for this experiment (Dharmacon). Forward primer sequence: CCGGGCATATGTGATGAGTGTAACCTCGAGTTACACTCATCACATATGCTTTTT. Reverse primer sequence: AATTAAAAAGCATATGTGATGAGTGTAACCTCGAGTTACACTCATCACATATG C. Oligos/primers were ordered from ThermoFisher Scientific. Briefly, a double restriction enzyme digest was performed with EcoRI and AgeI to release the stuffer from the pLKO-Tet-On parent plasmid. The digested vector was ligated with the shPHF5A #2 oligos with T4 DNA ligase (Cat# M0202S, New England Biolabs) and incubated for 2 hr at RT, then overnight at 16 °C. Ligation was transformed into NEB Competent cells per protocol and single clones were sequenced to ensure the oligos were incorporated correctly (**Figure 2-1**). Lentivirus was generated in Lenti-X 293T cells (Cat# 632180, Clontech) in 10 cm plates. To package the virus, the following was combined: 6 µg pLKO vector containing shPHF5A#2, 4.5 µg psPAX2 (gift from Didier Trono, Addgene plasmid #12260), and 3 µg pMD2.G (gift from Didier Trono, Addgene plasmid #12259) with FuGENE 6 per protocol (Promega) in Opti-MEM for transfection. The transfection

Sequence Name	< Pos = 240
- ■■■■ +	
☒ Consensus	ATATGTGATGAGTGTAAC TCGAGTTACACTCATCACATATGCTTTTAAATTCTCGAC
5 Sequences	40 250 260 270 280 290
full top primer num 2	- - - - - T GAGTGTAAC TCGAGTTACACTCATCACATATGCTTTT
JDH091	ATATGTGATGAGTGTAAC TCGAGTTACACTCATCACATATGCTTTTAAATTCTCGAC
JDH092	ATATGTGATGAGTGTAAC TCGAGTTACACTCATCACATATGCTTTTAAATTCTCGAC
JDH093	ATATGTGATGAGTGTAAC TCGAGTTACACTCATCACATATGCTTTTAAATTCTCGAC
JDH094	ATATGTGATGAGTGTAAC TCGAGTTACACTCATCACATATGCTTTTAAATTCTCGAC

Figure 2-1. Sequence Confirmation of shPHF5A Incorporation

Alignment of the forward primer sequence designed from siPHF5A #2 and 4 clones of the tet-pLKO-puro plasmid containing shPHF5A. Clones labeled JDH091-094. MegAlign software was used for alignment.

medium was replaced with fresh medium after 24 hr and viruses were collected after 48 hr. To remove cells and debris, the medium was filtered with a 0.45 µm PES filter, aliquoted, and frozen at -80 °C. For viral transduction, 1 ml of virus-containing medium mixed with 1 ml of fresh medium was added to a 6-well plate of AsPC-1 at 500 k cells/well with 8 µg/ml Sequa-brene (Cat# S2667, Sigma) overnight. The transduction medium was replaced with fresh medium containing 0.3 µg/ml puromycin dihydrochloride (Cat# P9620, Sigma) 24 hr later. The cells were grown in culture for 1 week to establish pooled puromycin-resistant stable cells, changing media every 2 days. To maintain the pooled stable cells for future experiments, 0.15 µg/ml of puromycin was used thereafter. For induction, the cells were grown in medium with tetracycline-free FBS (Clontech) before the addition of doxycycline. To induce shRNA expression in inducible stable cells, doxycycline was added to the culture medium

PHF5A Knock Out in AsPC-1 Cells

Glycerol stocks of 4 *PHF5A* guide RNAs were picked from the LentiArray Human Epigenetics CRISPR Library, Glycerol (Invitrogen). They are located in plate 10927, well B3 in rows 1-4, stored at -80 °C. Bacteria was streaked from glycerol stocks onto LB-ampicillin plates. Single colonies were picked and shaken overnight in 4 ml LB-amp broth at 37 °C at 200 RPM in a MaxQ 4450 incubated shaker (ThermoFisher Scientific). DNA was isolated from cultures using the QIAcube system (Qiagen). DNA was packaged into lentivirus via the protocol stated above. AsPC-1 Cas9-expression stable cells were transduced with the guide viruses AsPC-1. 24 hr later the media was changed. 48 hr after transduction, puromycin selection was initiated at 3 µg/ml, with a “no-transduction” control well. Media was changed every two days for six days to allow for recombination.

PXR Knock Out in AsPC-1 Cells

To generate a *PXR* knock out (KO) AsPC-1 cell line, the CRISPR/Cas9 sc-400824 and sc-400824-HDR plasmids were used (Santa Cruz Biotechnology, Inc.). The “CRISPR / Cas9 KO Plasmid and HDR Plasmid Transfection” protocol from Santa Cruz Biotechnology, Inc. (https://datasheets.scbt.com/protocols/CRISPR_protocol.pdf) was followed exactly, with the initial puromycin selection concentration being 3 µg/ml. Colonies were expanded from single clones in 48-well plates.

RNAseq

AsPC-1 cells were transfected in 6-well plates in triplicate with either lipid-only RNAiMAX control, siNT5, or siPHF5A pool. Reagent volumes are identical to the 6-well protocol found in the “siRNA Transfection” section above. siRNA was used at a final concentration of 25 nM. Total RNA was isolated from the AsPC-1 transfected cells, for isolation see “RNA Isolation and qPCR” section. The Nanodrop 8000 was used not

only to determine concentration of RNA, but to ensure purity of the RNA. Library prep was done by Dr. Rain Sun and RNAseq was performed by Dana Roeber on a HiSeq 4000 (Illumina). Resultant stranded paired-end 100-bp sequences were mapped to the hg19 human genome with the STRONGARM pipeline developed for the Pediatric Cancer Genome Project³⁴ and counted with HTSEQ. Statistical testing to determine differential expression was performed in R by using the voom and limma packages. By using our rnapeg in-house tool, exon junction reads were extracted to visualize alternative splicing and select differential junctions and to complement multivariate analysis of transcript splicing (MATS) 3.0.8 (python 2.7.2)³⁶. Principle component analyses was produced with Partek Genomics Suite 6.6. RNAseq initial analysis was performed by Dr. David Finkelstein.

Volcano Plot

Volcano plot was generated in Graphpad PRISM by plotting statistically significant, differentially expressed genes after removal of any genes affected by the siNT treatment. Statistically significant genes were plotted as a function of $-\log_{10}$ (adjusted p-value), and differential gene expression was plotted as a function of $\log_2$ (Fold change / siNT). Genes determined to be statistically significant and (1) down-regulated were highlighted in green, or (2) up-regulated were highlighted in red, as determined by $-\log_{10}(\text{p-value}) \geq 1$ and $\text{Log}_2(\text{FC}) \leq -1$ or ≥ 1 . Volcano plot was generated by William C. Wright.

CHAPTER 3. RESULTS

Genotyping CYP3A5

To understand which isoform(s) of CYP3A5 each cell line expresses, the following PDAC cell lines were genotyped: AsPC-1, HPAF-II, PANC-1, MIA PaCa-1, CFPAC-1, CAPAN-2, SU.86.86, and Panc02.13. Additionally, several other GI cancers and hTERT-HPNE cell lines were genotyped. HUH-7 cells were used as a control as this line is homozygous *CYP3A5**3/*3 (Dorr, 2017). The genotyping protocol was adapted from a protocol previously described (Saeki, 2003). All of the lines genotyped were homozygous *CYP3A5**3/*3 (G) at the site of the SNP (**Table 3-1**), however, HCT116 will need to re-genotyped as it was only performed once and is potentially a heterozygous at this site. Examples of electropherograms for each cell line are found in **Figure 3-1**. As CYP3A5*3 protein is not detectable or expressed, isoform-specific probes were utilized by Dr. Oghenechukome Lolodi to ensure that both CYP3A5*1 and CYP3A5*3 were expressed on the mRNA level in AsPC-1 cells. Bands were detected at both amplicon sizes, confirming the presence of both isoforms (data not shown). CYP3A5*1 protein can be detected via qPCR and western blotting in 6 of the 8 PDAC cell lines used in this study (**Figure 3-2A,C**). The samples in lanes 10 and 11 (**Figure 3-2C**) are from inducible CYP3A5 KD cells and overexpression stable AsPC-1 cell lines. They are included as expression controls and were provided by Dr. O. Lolodi.

Transcription Regulation of CYP3A5

To paint a clearer picture of what genes regulate CYP3A5 expression, a high-throughput screen was designed. The AsPC-1 PDAC cell line was chosen as a screening model because it has high basal CYP3A5, PXR, and HNF4A expression (Rouillard, 2016), which was confirmed via RNAseq by Dr. Peter Oladimeji (data not shown).

First, a published regulator of basal *CYP3A5* expression, HNF4A, was tested in AsPC-1 cells (Noll, 2016). Cells were transfected with siRNA targeting HNF4A and/or PXR, since PXR is downstream of HNF4A. It was found that at 72 hr post-transfection neither siHNF4A nor siPXR decrease basal CYP3A5 levels at the mRNA or protein levels in AsPC-1 cells, as measured by western blotting and qPCR (**Figure 3-3A-B**). Knock down efficiency was greater than 70% for the putative regulators: PHF5A (**Figure 3-3C**), HNF4A (**Figure 3-3D**), and PXR (**Figure 3-3E**). Next, this observation was tested in 5 other ATCC PDAC cell lines: HPAF-II, Panc02.13, SU.86.86, CFPAC-1, and CAPAN-2. In all cases, CYP3A5 levels are unchanged when HNF4A and PXR are knocked down. This is shown via western blotting (**Figure 3-4A-D**) and qPCR (**Figure 3-4E-I**) for each cell line. Western blot data is not shown for CAPAN-2 as CYP3A5 protein expression was undetectable, therefore, only qPCR data is shown (**Figure 3-4H**).

Table 3-1. *CYP3A5* Genotyped Cell Lines

Cell Line	Cell Type	Genotype
AsPC-1	PDAC	G/G
HPAF-II	PDAC	G/G
PANC-1	PDAC	G/G
MIA PaCa-1	PDAC	G/G
CFPAC-1	PDAC	G/G
CAPAN-2	PDAC	G/G
Su.86.86	PDAC	G/G
PANC02.13	PDAC	G/G
hTERT-HPNE	“normal” ductal	G/G
HUH-7	HCC	G/G
LS180	CRC	G/G
SNU-C4	CRC	G/G
HCT 116	CRC	A/G*
HepG2-C3A	HCC	G/G

*needs further testing

Figure 3-1. CYP3A5 rs776746 SNP Electropherograms

Electropherograms revealing the genotype of CYP3A5 regarding the 6986A>G SNP in 8 PDAC cell lines and 6 other cell lines.

Figure 3-2. CYP3A5 and PHF5A Expression in 8 PDAC Cell Lines

A. qPCR data showing CYP3A5 relative expression, plotted as $2^{-\Delta\text{Ct}}$. B. qPCR data showing PHF5A relative expression, plotted as $2^{-\Delta\text{Ct}}$. C. Western blot of CYP3A5, PHF5A, and β -actin primary antibodies. Lanes 10 and 11 contain inducible CYP3A5 KD and expression constructs, respectively, provided by Dr. O. Lolodi. 45 μg protein loaded per lane.

Figure 3-3. siPHF5A, but Not siPXR/siHNF4A, Affects Basal CYP3A5 Expression in AsPC-1 Cells

AsPC-1 cells were treated with a final concentration of 25 nM for 72 hr. A. Western blot of CYP3A5 and β -actin primary antibodies. 50 μ g protein loaded per lane. B-E. Fold change represents $2^{\Delta\Delta Ct}$, normalized to GAPDH of siNT5. B. CYP3A5 expression. C. PHF5A expression. D. HNF4A expression. E. PXR expression.

Figure 3-4. siPHF5A, but Not siPXR/siHNF4A, Affects Basal CYP3A5 Expression in 5 PDAC Models

PDAC lines, HPAF-II, Panc02.13, SU.86.86, CFPAC-1, and CAPAN-2, were treated with a final concentration of 25 nM for 72 hr. A-D. 50 µg protein loaded per well. Western blot of CYP3A5, PHF5A, and β-actin primary antibodies. Lane labels of siRNA in A apply to B-D as well. E-I. qPCR results for CYP3A5, PHF5A, PXR, and HNF4A using ddCt method. WB data for CFPAC-1 not shown.

To determine what factors affect *CYP3A5* expression in PDAC cell lines, an siRNA screen was performed. Before running the screen, a series of experiments was undertaken to optimize screening conditions. To confirm the mRNA and protein levels of *CYP3A5* in AsPC-1 and the knockdown efficiency of the siRNA, cells were seeded into 6-well plates, reverse transfected with control siNT4 and siCYP3A5, and collected 48 hr later. siRNA was used at a final concentration of 25 nM. Lysed samples were analyzed via qPCR and western blotting to detect knockdown of *CYP3A5*. *CYP3A5* was detectable at both the mRNA and protein levels, and expression was successfully knocked down up to 70% via qPCR (**Figure 3-5A**) and western blotting (**Figure 3-5B**).

To select the most desirable non-targeting siRNA control, an siNT optimization experiment was performed. AsPC-1 cells were reverse transfected with siNT1, siNT2, siNT3, siNT4, and siNT5, and 48 hr later examined via measuring *CYP3A5* immunofluorescence intensity (**Figure 3-5C**). siRNA was used at a final concentration of 25 nM. Results highlighted the range of variability that “non-targeting” controls can exhibit. siNT2 and siNT5 were found to behave similarly to lipid controls, therefore, siNT5 was chosen as the NT control for all future experimentation.

To obtain maximum transfection efficiency to ensure a good fluorescence signal window, a lipid optimization experiment examined the efficiency of the lipids: RNAiMAX, red Dharmafect, and blue Dharmafect. Four wells per treatment were collected to analyze gene expression. Immunofluorescence showed that RNAiMAX and the blue Dharmafect yielded slightly better *CYP3A5* signal knockdown than red Dharmafect, so RNAiMAX was selected for use in future experiments (**Figure 3-5D**). Representative images of RNAiMAX-transfected cells are shown in **Figure 3-5E**.

To determine what timepoint yielded superior knockdown of *CYP3A5*, a comparison siRNA experiment was performed to test KD at 48 and 72 hr. It was found that *CYP3A5* mRNA levels are more efficiently decreased at 72 hr (**Figure 3-5F**).

To optimize the dilution of primary *CYP3A5* antibody used for screening, an antibody titration experiment was performed. A serial dilution from 1:100 to 1:8000 was used and plates were scanned for *CYP3A5* immunofluorescence. The amount of antibody used greatly affected the signal window and intensity, with 1:100 having the greatest intensity, but the middle range dilutions having better separation between siNT5 and siCYP3A5 treatments, therefore, a dilution of 1:400 was selected for the final screening conditions (**Figure 3-5G**).

Once conditions were optimized, miniaturization to 384-well from 96-well plates was undertaken. To ascertain the appropriate cell density of AsPC-1 cells in a 384-well plate, cells were seeded with decreasing cell number and incubated for 72 hr. Using the IncuCyte Zoom, endpoint images were taken and it was determined that 6,000 cells/well is the optimal number of cells for immunostaining AsPC-1 (**Figure 3-5H**).

To address *CYP3A5* regulation, a high-throughput siRNA screen of ~1,500 transcription factors and chromatin-associated proteins was performed in the AsPC-1

Figure 3-5. High-Throughput siRNA Screening Condition Optimization

A-B. AsPC-1 cells transfected with 10 nM final concentration control siNT4 or siCYP3A5 for 48 hr. A. Gene expression was analyzed via qPCR normalized to GAPD using the ddCt method. B. Western blot of CYP3A5 and β -actin primary antibodies. C. AsPC-1 cells were treated with 10 nM final concentration siNT1-5 for 48 hr and stained with CYP3A5 primary antibody. 24 fields per treatment were analyzed using the InCell Analyzer. D-E. AsPC-1 cells were reverse transfected with a final concentration of 25 nm siRNA to CYP3A5, NT4, and a lipid-only control. Lipids tested were RNAiMAX, red Dharmafect, and blue Dharmafect. D. CYP3A5 fold change over GAPDH normalized to siNT4. E. Cells were stained with primary anti-CYP3A5 antibody (1:75), secondary Alexa Fluor 647 (1:1000), DAPI, and Phalloidin-488 (1:1000). 48 hr timepoint. 20X representative images from RNAiMAX-transfected cells. Images displaying red channel (CYP3A5 staining). Image E1. Lipid only control, E2. siNT4, and E3. siCYP3A5. F. AsPC-1 cells were reverse transfected with 25 nm siRNA to CYP3A5 and siNT5. Fold change over GAPDH normalized to siNT5 control. Cells were collected 48 and 72 hr post-transfection. CYP3A5 fold change is shown. G. AsPC-1 cells were reverse transfected with 25 nm siRNA to CYP3A5 and siNT5. Cells were stained with primary anti-CYP3A5 antibody in a serial dilution, secondary Alexa Fluor 647 (1:1000), DAPI, and Phalloidin-488 (1:1000). 12 fields per well were analyzed. Shown is the normalized CYP3A5 intensity. H. AsPC-1 cells were seeded into a 384-well plate and incubated for 72 hr. 10X images and confluence percentages taken by IncuCyte Zoom 77 hr post-plating. Average confluence of 6 well per density is shown: (1). 6K cells/well, average confluence 30.4% (2). 4K cells/well, average confluence 18.9% (3). 2K cells/well, average confluence 11.4% (4). 1K cells/well, average confluence 4.6%.

cell line. The screen's readout was CYP3A5 immunofluorescence, with the desired result being the identification of putative positive regulators. Positive regulatory hits would have decreased CYP3A5 signal upon KD of the aforementioned regulators. Optimizations were carried out before the screen to determine the following: cell number, primary antibody concentration, suitable secondary, secondary antibody concentration, suitable whole-cell stain, stain concentration, conditions for fixation, blocking, washing, permeabilization, and transfection duration, key points shown in **Figure 3-5**.

Screen Analysis

Once the screen was completed, Dr. Yueming Wang scanned each of the 18 plates according to an InCell analysis protocol. Briefly, cell location was determined by DAPI staining and object segregation. Cell number/area was determined by phalloidin (green channel) and object segregation. Total CYP3A5 level was determined by the average intensity of CYP3A5 (red channel) per cell in each well multiplied by the total area. This total calculated CYP3A5 level was then divided by total cell area to achieve normalized CYP3A5 level. This normalized total CYP3A5 level is used for statistical analysis and quality control. An example of this masking is found in **Figure 3-6A**. This analysis yielded strictly standardized mean difference (SSMD) values which were used for QC and hit selection (**Figure 3-6B**).

Hit Selection and Top 20 Hits

Based on SSMD, visual downregulation, and cell number, the following 20 hits of interest were selected: PHF5A, SMARCD1, ZNF354A, BARX2, CDX2, GTF3C1, ZNF397, ZFH3, KDM5A, PPARG, CREBBP, GATA3, HIF1A, MED17, RNF112, KLF13, SLC4A10, TFAP4, CDY2A, and NOTCH2. Pooled siRNA was tested for each of the 20 hits and the subsequent CYP3A5 levels were detected via western blotting (**Figure 3-7A-D**) and qPCR (**Figure 3-7E-F**). Many of the top 20 hits were validated as positive regulators of CYP3A5 expression, meaning that that when the gene of interest was knocked down, CYP3A5 levels correspondingly decreased. This indicates that the screen produced a good set of hits in line with the desired phenotype.

Top 5 siRNA Deconvolution

Of the top 20 hits that were validated as positive regulators, 5 were chosen for siRNA deconvolution: PHF5A, BARX2, ZNF354A, CDX2, and SMARCD1. siRNA deconvolution ensures that the best individual siRNAs producing maximal KD are identified for future use. AsPC-1 cells were reverse transfected with a final concentration of 25 nM siRNA, with each gene having 4 individual siRNAs (**Figure 3-8**). Upon deconvolution it was found that the individual siRNAs targeting PHF5A yielded greatest overall KD of CYP3A5 mRNA levels, as determined by qPCR (**Figure 3-8A**). The other 4 genes, upon deconvolution, only had one siRNA each that resulted in CYP3A5 levels

Figure 3-6. siRNA Screen Analysis

A. Example of InCell Analyzer masking. Yellow indicates CYP3A5 staining, green indicates phalloidin-488 staining, and blue indicates DAPI nuclei staining B. Waterfall plot ranking siRNAs according to SSMD.

Figure 3-7. Top 20 Screen Hit Confirmation

siRNA directed to each of the top 20 screen hits was reverse transfected into AsPC-1 cells for 72 hr at a final concentration of 25 nM. A-D. Western blots of the 20 hits for CYP3A5 and β -actin antibodies. 40 μ g protein loaded per well. E-F. qPCR showing fold change (ddCt over GAPDH) for CYP3A5.

Figure 3-8. Top 5 Screen Hit siRNA Deconvolution

A-E. siNT5, siCYP3A5, and individual siRNA targeting the top 5 hits were reverse transfected into AsPC-1 cells for 72 hr at a final concentration of 25 nM. CYP3A5 fold change and gene-specific expression are shown for A. PHF5A, B. BARX2, C. ZNF354A, D. CDX2, and E. SMARCD1.

decreasing below 50% as compared to control siRNA (**Figure 3-8B-E**). A second replicate of siPHF5A deconvolution was done to verify findings and to compare pooled siRNA to individual. It was confirmed that all individual siRNA lead to CYP3A5 mRNA decreases below 50%, with siPHF5A #2 and #18 mimicking the efficacy of the pooled siRNA (**Figure 3-9A**). For each treatment, the PHF5A KD efficiency was excellent, indicating good transfection efficiency and destruction of the target mRNA (**Figure 3-9B**). A western blot was performed on these same samples, and there was a corresponding decrease of CYP3A5 protein upon PHF5A KD for both pooled and individual siRNA (**Figure 3-9C**).

This project subsequently centered on the top hit of interest, PHF5A, due to the observed phenotype upon KD. It is unknown how siPHF5A leads to decreased CYP3A5 mRNA and protein levels. A time-course of PHF5A siRNA was performed to determine optimal transfection conditions and to identify the window in which siPHF5A is correlated with a maximal decrease in CYP3A5 levels. The timepoints were 24, 48, and 72 hours and it was found that siPHF5A had a time-dependent effect on CYP3A5 mRNA levels (**Figure 3-10A**). PHF5A KD efficiency was similar in all timepoints (**Figure 3-10B**). Confirmation of this phenotype was shown in AsPC-1 cells (**Figure 3-3A-C**) and in 5 other PDAC lines via western blot (**Figure 3-4A-D**, lane 3) and qPCR (**Figure 3-4E-I**).

The PAF1C as a Potential PHF5A Mechanism

After hit validation, the next step was to undertake mechanistic studies to elucidate how the KD of PHF5A is related to the downregulation of CYP3A5. By examining expression levels of CYP3A5 and PHF5A and experimental data, it was clear that PHF5A and CYP3A5 basal levels do not correlate (**Figure 3-2A-C**). This indicates that another protein or proteins may be involved. One published mechanism PHF5A could be working through to affect CYP3A5 is the PAF1C. When using siRNA to knock down individual members of the PAF1C, there is minimal or no effect on CYP3A5 levels (**Figure 3-11**). Since the disruption of one complex member could potentially be compensated for by the others. Double knock down samples addressed this issue and it was found that when knocked down in combination, PAF1 and WDR61 seem to be necessary for full CYP3A5 expression (**Figure 3-11**). Interestingly, CDC73 seems to have a protective role by rescuing the decrease in CYP3A5 mRNA levels by KD of other components of the PAF1C. Further studies may shed light on the intricacies of the mechanism at play with the PAF1C/CYP3A5 relationship.

Splicing as a Potential PHF5A Mechanism

Another name for PHF5A is SF3b14b, named from its role as a member of the SF3B subcomplex of the U2 snRNP of the spliceosome. Since CYP3A5 levels decrease when PHF5A is knocked down, the next step was to manipulate key members of this

Figure 3-9. Deconvolution of siPHF5A in AsPC-1 Cells

A-C. siNT5, siCYP3A5, and pooled and individual siRNA targeting PHF5A were reverse transfected into AsPC-1 cells for 72 hr at a final concentration of 25 nM. A-B. qPCR showing fold change (ddCt over GAPDH) A. CYP3A5 expression. B. PHF5A KD efficiency. C. Western blots for CYP3A5 and β -actin antibodies. 40 μ g protein loaded per well.

Figure 3-10. siPHF5A Time Course

A-B. AsPC-1 cells were reverse transfected with siRNA targeting CYP3A5 and PHF5A, with an siNT5 control, and cells were collected at 24, 48, and 72 hr post-transfection. A. CYP3A5 fold change B. PHF5A KD efficiency.

Figure 3-11. siPAF1 and siWDR61 Affect CYP3A5 Expression

AsPC-1 cells were reverse transfected with siRNA targeting each of the 6 members of the PAF1C, individually or in combination and collected 72 hr later for qPCR. Each siRNA had a final concentration of 25 nM, therefore, in lanes 1-9, and equal proportion of siNT5 was supplemented to make the total siRNA concentration the same as double KD lanes. A total of 50 nM siRNA was present in each well.

complex to assess their involvement and investigate if this is a PHF5A-specific effect. It was found that knocking down SF3B1 and SF3B3 does lead to a corresponding decrease in CYP3A5 protein (**Figure 3-12A**), but not mRNA (**Figure 3-12B**). Neither individual or dual KD of these splicing factors decreased CYP3A5 levels to the extent that KD of PHF5A did.

Using siRNA to target members of the spliceosome addresses the role of splicing in CYP3A5 regulation from a genetic standpoint. Addressing this issue from a pharmacological angle added confidence to this mechanistic study. Preliminary results suggest that treating AsPC-1 cells with the splicing inhibitor Pladienolide B leads to decreased levels of CYP3A5 (**Figure 3-13A**), mimicking the effect of PHF5A KD. A proliferation assay established the standard growth curve in AsPC-1 for control treated cells (**Figure 3-13B**). A range of Pladienolide B and Herboxidiene concentrations were tested in AsPC-1 to determine toxic concentrations (**Figure 3-13C-D**). Initial findings suggest that there are concentrations in which the drug is not toxic to the cells. These data will need to be repeated and the drug-treated cells analyzed, as they were only carried out once.

RNAseq Reveals siPHF5A Influence

To understand the scope of *PHF5A* influence in the AsPC-1 cell line, RNAseq of siPHF5A vs siNT5 and lipid-only transfected cells was performed. qPCR was performed before the samples were sent for sequencing to ensure good KD of PHF5A and CYP3A5 (**Figure 3-14A**). The sequencing core determined RIN values for each of the samples before running the RNAseq as a quality control measure (**Figure 3-14B**). RIN is a metric offered by the Agilent Bioanalyzer as an estimate of the extent of degradation of total RNA. An ideal value is close to 10, and experimental values ranged from 9.6 to 10. Briefly, the data produced by RNAseq of the siPHF5A samples produced two types of data: splicing event data and gene expression data. A member of the bioinformatics core, Dr. Finkelstein, performed MATS analysis to determine the number, frequency, and identity of the alternative splicing events between the two data sets, siPHF5A vs siNT5 (**Figure 3-14C**). William C. Wright, did a thorough analysis of the differential gene expression dataset. Preliminary conclusions are that PHF5A KD affects a host of other genes besides *CYP3A5*, including *MUC17* and *GFAP* (**Figure 3-14D**). Alternative splicing can also be assessed with this dataset, and more detailed analysis is needed in this area. Future validation and investigation will need to be undertaken to understand the scope of PHF5A's effect on drug metabolism, splicing, and other pathways in AsPC-1 cells.

Tools to Study PHF5A and CYP3A5

Thus far, siRNA has been employed to investigate the effects of knocking down published and putative regulators of *CYP3A5*. Three stable cell lines were created from

Figure 3-12. siSF3B Affects CYP3A5 Protein but Not mRNA

A-B. siNT5 and pooled siRNA targeting CYP3A5, PHF5A, SF3B1, and SF3B3 were reverse transfected into AsPC-1 cells for 72 hr at a final concentration of 25 nM each, 50 nM total concentration as siNT5 was added to single KD samples to make the total siRNA concentration equal. A. Western blot for CYP3A5, PHF5A, SF3B1, and β -actin antibodies. 40 μ g protein loaded per well. B. qPCR showing fold change (ddCt over GAPDH) for CYP3A5.

Figure 3-13. Splicing Inhibitors in AsPC-1 Cells

A. AsPC-1 cells were treated with Pladienolide B and a western was done with CYP3A5, PHF5A, SF3B1, and β -actin antibodies. 40 μ g protein loaded per well. B-D. AsPC-1 cells were seeded into 96-well plates, treated with compound and IncuCyte images were taken every 6 hr for 96 hr. Time zero is time of drugging. Confluence was measured with IncuCyte software. B. Growth curve of AsPC-1 cells in 0.1% DMSO. C. AsPC-1 cells were treated with decreasing concentrations of Pladienolide B. D. AsPC-1 cells were treated with decreasing concentrations of Herboxidiene.

Figure 3-14. RNAseq Analysis of siPHF5A Samples

AsPC-1 cells were transfected with lipid only, siNT5, or siPHF5A pool and submitted in triplicate for RNAseq analysis. A. RNAseq qPCR validation before submitting samples, CYP3A5 fold change is shown. B. RIN mRNA degradation estimates for each RNAseq sample. Generated by Dana Roeber. A1-C1 are lipid only, D1-F1 are siNT5, and G1-A2 are siPHF5A. C. Summary of the number and type of statistically significant alternative splicing events, as determined by multivariate analysis of transcript splicing (MATS) (false discovery rate <0.05). A3SS, alternative 3' ends; A5SS, altered 5' ends; SE, cassette exons; and RI, retained introns. D. Gene expression volcano plot generated in PRISM by William C. Wright.

parental AsPC-1 cells to add a layer of confidence to this study and enable mechanistic studies.

To provide better control of *PXR* expression/induction, a two-plasmid CRISPR system was employed to knock out *PXR* in AsPC-1, the original pool displayed about a 50% decrease in *PXR* fold change (**Figure 3-15A**). Since this should be a KO, not a KD, cells were expanded from single clones to identify clones which had KO of *PXR* expression (**Figure 3-15A**).

An inducible KD system was used to generate inducible shPHF5A #2 to allow temporal control over PHF5A KD. Cloning was done to insert the shPHF5A primers into the pLKO parent plasmid. AsPC-1 cells were transduced and selected with puromycin to isolate a population of stable cells that can be induced to KD PHF5A upon doxycycline treatment. A doxycycline dose response was undertaken, and will need to be repeated as it was performed only once. At all concentrations of doxycycline, PHF5A was knocked down by about 40% and CYP3A5 accordingly decreased at some lower concentrations (**Figure 3-15B**).

To study the effects of *PHF5A* KO in AsPC-1, four PHF5A-specific guide RNAs (gRNAs) were transduced in AsPC-1 cells stably expressing Cas9. The cells containing the two gRNAs that grew well after puromycin selection were expanded and collected for downstream analysis. CYP3A5 levels were mildly decreased via qPCR (**Figure 3-15C**), but not changes were detected via western blotting (**Figure 3-15D**).

Figure 3-15. Tools Built for PHF5A Mechanistic Studies

PXR expression in PXR CRISPR KO AsPC-1 cell line. B. CYP3A5 and PHF5A expression in tet-on-pLKO-puro shPHF5A AsPC-1 stable cells when induced with decreasing concentration of doxycycline. C-D. PHF5A KO AsPC-1 cell line (pool). C. CYP3A5 and PHF5A expression D. Western of CYP3A5, PHF5A, and β -actin antibodies. 40 μ g protein loaded per lane.

CHAPTER 4. DISCUSSION

The initial goal of this project was to investigate the regulation of *CYP3A5* in a pancreatic cancer cell line via an siRNA screen knocking down transcription factors and chromatin-associated proteins, and to follow up on the regulatory mechanism of top hit PHF5A. This led to initial probing into different potential mechanisms by which PHF5A could be acting on *CYP3A5*. These studies were initiated in the AsPC-1 PDAC cell line because of its high basal expression of *CYP3A5* that can be knocked down with siRNA at both the mRNA and the protein level (**Figure 3-5A-B**). Many of the mechanistic studies will need to be repeated and expanded, but initial findings are promising, pointing to PHF5A's role in splicing.

Three stable cell lines were created in order to assist future work on this project (**Figure 3-15A-D**). These include a *PXR* KO AsPC-1 cell line, a doxycycline-inducible shPHF5A AsPC-1 cell line, and a *PHF5A* KO AsPC-1 line. These tools can approach the question of *CYP3A5* regulation by offering knocked out regulators and temporal control of PHF5A KD. To determine if both alleles were affected by the CRISPR KO, PCR amplification and sequencing of the gene will need to be done.

This project also brought to light the need to better understand *CYP3A5* genotype and the role this plays. The fact that homozygous *CYP3A5**3 individuals express some active *CYP3A5* (Kuehl, 2001) indicates that *CYP3A5**3 is a leaky allele. This is supported by the data that shows full length *CYP3A5* is detected in all *CYP3A5*-expressing cell lines (**Figure 3-1**). Understanding how the *CYP3A5**3 allele is generated could lead to the ability to correct this genotype to alter phenotype, which could lead to drug-sensitized cancers. The rs776746 SNP creates a cryptic acceptor splice site that promotes the insertion pseudoexon 3B in the mature mRNA. This improperly spliced mRNA contains a premature stop codon, preventing full length *CYP3A5* protein from being made. Remember, *CYP3A5**3 homozygous individuals produce some active *CYP3A5*, reinforcing that the splicing machinery has a choice of acceptor sites (Lee, 2007). The SNP occurs over 100 base pairs upstream from the splice donor site of exon 4, and currently there are no models to explain how normal splicing is impaired but not inhibited (Annalora, 2017). More in-depth studies need to be done to characterize this site selection.

To answer the outstanding questions of this project, future work can be done using isoform-specific probes to determine which isoform(s) of *CYP3A5* are expressed in each cell line, which are affected by siPHF5A, and how this affects drug metabolism.

All 8 PDAC lines in this study have the same genotype, but PANC-1 and MIA PaCa-1 are not abundant in *CYP3A5* mRNA or protein (**Figure 3-1A,C**). So, what makes the expression profile different? Differential regulation? Co-occurring mutations? Not only is this an interesting basic biological question, it is also applicable in pharmacogenomics. Understanding a patient's *CYP* SNP panel would further the field of personalized medicine and improved therapeutics.

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