Improving Platinum-Based Treatments for Advanced Non-Small Cell Lung Cancer

by

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Abstract

Novel treatments are urgently needed for patients with non-small cell lung cancer (NSCLC). Currently, these patients are almost always first treated with cisplatin (CDDP)-containing drug combinations. To identify therapeutic targets that could enhance CDDP activity, a genome-wide siRNA screen looking for synthetic lethal partners with low-dose CDDP was completed. These data were combined with results from a microarray study assessing differentially expressed genes in NSCLC cells exposed to low-dose CDDP. The results indicated that 151 genes were differentially expressed in cells exposed to low-dose CDDP. Nine up-regulated genes ranked within the top 10% of the siRNA screen based on a scoring method that considered minimal loss of cell viability from gene knockdown alone and significant enhancement of CDDP activity. Five genes were further validated and two (*RRM2B* and *CABYR*) were found to significantly improve the cytotoxic effects of CDDP. Pathways involved in repairing double-stranded DNA breaks and INO80 chromatin remodeling were enriched in both datasets. Analysis of the kinome subset of the siRNA screen also identified PAPSS1 (3’-phosphoadenosine 5’-phosphosulfate synthase 1) as a protein that when silenced sensitized NSCLC cells to CDDP. PAPSS1 produces the biologically active form of sulfate for sulfonation reactions. PAPSS1-silencing combined with low-dose CDDP reduced the clonogenicity of NSCLC cells by 98.7%, increased DNA damage, and induced G1/S phase cell cycle arrest. PAPSS1 suppression also sensitized NSCLC cells to radiation and topoisomerase I inhibitors. Sensitization was cancer cell specific. The extent of CDDP potentiation increased substantially when NSCLC cells were stressed by starvation or hypoxia. In NSCLC cell spheroids and zebrafish xenografts, PAPSS1 silencing in combination with CDDP decreased tumor size, while the same dose of CDDP combined with non-silencing controls led to increases in tumor size. In a subcutaneous tumour model, expression of PAPSS1-targeting shRNA in combination with a non-curative dose of CDDP enhanced activity compared to controls. Future studies are needed to identify small molecule inhibitors and proteins that interact with PAPSS1. These tools will be useful to fully understand the mechanisms by which chemosensitization occurs and such tool compounds may prove useful as therapeutics that would benefit NSCLC patients when first treated.
Preface

The research program was originally designed by Dr. Marcel Bally and co-applicants (Drs. Mohammad Qadir, Sam Aparicio, Marco Marra, Steven Jones, and Janessa Laskin) of a Canadian Institutes of Health Research funded operating grant (MOP 89948). The genome-wide siRNA screen and the microarray study described in the thesis were conducted prior to the start of my graduate studies, but the data were not yet analyzed. The statistical analyses of the screen data were performed by Drs. Steven McKinney and Steven Poon. I led efforts towards the identification and validation of gene targets by analyzing datasets and designing as well as conducting validation experiments. I was responsible for analyzing data and preparing all manuscripts included in this Thesis.

Chapter 1 includes background information on lung cancer and a literature review on synthetic lethality:

Leung AW, De Silva, T, Bally, MB, Lockwood, W. Synthetic Lethality in Lung cancer and Translation to Clinical Therapies.

This work is submitted as a review article and was completed in collaboration with Dr. William Lockwood and Tanya de Silva. Parts of the section on “Types of Synthetic Lethality and Identified Interactions in Lung Cancer” were originally drafted by Dr. Lockwood and I have edited this text to highlight key points relevant to my thesis project. I organized the manuscript, wrote the vast majority of the text and designed all the figures.

The research described in Chapter 2 has been published:


Analysis of the microarray was performed by Dr. Stacy Hung (bioinformatician) and the yeast studies were completed by Dr. Peter Stirling’s laboratory at the Terry Fox Laboratories (BC Cancer Research Centre). The methods and results from the yeast studies were originally drafted by Dr. Peter Stirling. I designed the systematic analysis and overlay of the two datasets (siRNA screen and microarray study) and conducted experiments necessary to validate the microarray results. I selected the gene targets and designed the validation studies which were performed by Ian Backstrom under my supervision. I wrote
most of the manuscript as well as prepared all figures and tables. I was responsible for working with the PLoS ONE editor to address reviewer concerns.

The research described in Chapter 3 has been published:


The siRNA screen and initial validation of the top kinases were completed by Brian Kwok, Daniel Ricaurte, and Amith Ahluwalia. I designed, performed, and analyzed all validation studies on the gene target PAPSS1. Experiments involving flow cytometry were designed, conducted, and analyzed by myself with expert guidance from Wieslawa Dragowska. Yeast studies were performed by Dr. Peter Stirling’s laboratory. The supplemental studies in yeast were originally drafted by Drs. Veena Mathew and Peter Stirling. I wrote the manuscript, prepared all figure and tables and responded to reviewer’s concerns as required for publication.

The research described in Chapter 4 is submitted:

**Leung AWY**, Veinotte, C, Backstrom, I, Parsons, K, Bernardi, E, Warburton, C, Yapp, DTT, Berman, J, and Bally, MB. In Vivo Validation of PAPSS1 (3’phosphoadenosine 5’-phosphosulfate synthase 1) as a Cisplatin-Sensitizing Therapeutic Target in Non-Small Cell Lung Cancer.

Zebrafish studies were performed in Dr. Jason Berman’s laboratory (Dalhousie University). The methods and results section on the zebrafish studies were originally drafted by Chansey Veinotte and then edited by me to be consistent with the manuscript. Spheroid studies were optimized by Ian Backstrom. The shRNA-modified cell line was generated by me with expert guidance from Dr. Corinna Warburton. I designed, performed, and analyzed all *in vitro* experiments. I designed and prepared the cells and test articles for the murine studies and the animal work was conducted by the animal technicians from the Investigational Drug Program at the BC Cancer Agency. I wrote the manuscript and prepared the figures and tables.

Chapter 5 includes a discussion on translating results from synthetic lethality studies to clinical applications. This is part of the review article also mentioned in Chapter 1. I wrote
the text prepared for Chapter 5 and prepared all figures. Chapter 5 also includes a literature review on sulfonation reactions which is accepted by Oncotarget:

Leung AW, Backstrom, I, and Bally, MB. Sulfonation, an Underexploited Area: From Skeletal Development to Infectious Diseases and Cancer.

I wrote the manuscript and prepared the figures. The validation studies in ovarian cancer were performed by Ian Backstrom under my supervision.

The supplemental data included in Appendix A are part of the manuscripts described above. I was responsible for collecting and presenting these data.

All manuscripts were reviewed and edited by my supervisor Dr. Marcel Bally.

The scientific research presented in this thesis was completed in accordance with the University of British Columbia Policies and Procedures, Biosafety Practices and Public Health Agency of Canada guidelines (Biohazard Approval Certificate Protocols B08-0113, B10-077, B14-0241). All rodent studies were completed under an animal care protocol (A14-0290) approved by the Institutional Animal Care Committee (IACC). The IACC for studies conducted at the BC Cancer Agency (Vancouver Cancer Research Centre) Animal Resource Center is operated by the University of British Columbia in accordance to the Canadian Council of Animal Care (CCAC).
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<th>Description</th>
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<tbody>
<tr>
<td>ALDH3A1</td>
<td>Aldehyde dehydrogenase 3 family member A1</td>
</tr>
<tr>
<td>BRCA</td>
<td>Breast Cancer Susceptibility Protein</td>
</tr>
<tr>
<td>CABYR</td>
<td>Calcium binding tyrosine phosphorylation regulated protein</td>
</tr>
<tr>
<td>CBDCA</td>
<td>Cyclobutane Dicarboxylic Acid (Carboplatin)</td>
</tr>
<tr>
<td>CDDP</td>
<td>Cis-Diamminedinchloridoplatinum (II) (Cisplatin)</td>
</tr>
<tr>
<td>CS</td>
<td>Chondroitin Sulfate</td>
</tr>
<tr>
<td>CSPG</td>
<td>Chondroitin Sulfate Proteoglycan</td>
</tr>
<tr>
<td>DRC</td>
<td>Dose Response Curve</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>FHL2</td>
<td>Four and a Half LIM domains 2</td>
</tr>
<tr>
<td>GBM</td>
<td>Glioblastoma Multiforme</td>
</tr>
<tr>
<td>GOF</td>
<td>Gain-of-Function</td>
</tr>
<tr>
<td>HBEp</td>
<td>Human Bronchial Epithelial</td>
</tr>
<tr>
<td>Hh</td>
<td>Hedgehog (Pathway)</td>
</tr>
<tr>
<td>HLMVEC</td>
<td>Human Lung Microvascular Endothelial Cells</td>
</tr>
<tr>
<td>HR</td>
<td>Homologous Recombination</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan Sulfate</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan Sulfate Proteoglycan</td>
</tr>
<tr>
<td>IC&lt;sub&gt;10&lt;/sub&gt;</td>
<td>Inhibitory Concentration of 10%</td>
</tr>
<tr>
<td>KRAS</td>
<td>Kirsten Rat Sarcoma Viral Oncogene Homolog</td>
</tr>
<tr>
<td>LAC</td>
<td>Lung Adenocarcinoma</td>
</tr>
<tr>
<td>LOF</td>
<td>Loss-of-Function</td>
</tr>
<tr>
<td>MDS</td>
<td>Multidimensional Scaling</td>
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</table>
NER  Nucleotide Excision Repair  
NINJ1  Nerve Injury-Induced Protein 1  
NLS  Nuclear Localization Signal  
NOEL  No-Observed-Effect Level  
NSCLC  Non-Small Cell Lung Cancer  
PAPS  3’-Phosphoadenosine 5’-Phosphosulfate  
PAPSS  3’-Phosphoadenosine 5’-Phosphosulfate Synthase  
PE  Plating Efficiency  
PKS  Preliminary Kinome Screen  
PLK1  Polo-Like Kinase 1  
PTEN  Phosphatase and Tensin Homolog  
PTS  Protein Tyrosine Sulfonation  
RMA  Robust Multi-Array Average  
RNAi  RNA Interference  
RRM2B  Ribonucleotide Reductase M2 B  
RT-qPCR  Real-Time-Quantitative Polymerase Chain Reaction  
SCLC  Small Cell Lung Cancer  
SF  Surviving Fraction  
shRNA  Short Hairpin RNA  
siRNA  Small Interfering RNA  
SqCC  Squamous Cell Carcinoma  
SULT  Sulfotransferase  
TPST  Tyrosylprotein Sulfotransferase  
TPT  Topotecan  
WGS  Whole Genome Screen  
WT  Wild-Type
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Special thanks to my family for their unconditional and extraordinary moral support throughout the degree.
To my supervisor Dr. Marcel Bally
1. Introduction

1.1 Project Overview, Hypothesis, and Aims

Non-small cell lung cancer (NSCLC) attributes to over 80% of lung cancers with most patients diagnosed with a locally advanced or metastatic disease [1, 2]. The standard of care for patients with no targetable driver mutations is platinum-based combination chemotherapy comprising a platinum agent (cisplatin/carboplatin) and a selected second cytotoxic drug (e.g. vinorelbine, paclitaxel, etoposide). In cases where the patient may benefit from targeted therapy, platinum-based doublets are still used either in combination with the targeted treatment (e.g. Bevacizumab, Cetuximab) or as a second line treatment. Hence, platinum-based combination chemotherapy remains the cornerstone treatment for managing advanced NSCLC. Although platinum-based treatments only yield a 30-33% response rate, these treatments provide significant benefits for the patients and will not be replaced in the near future [3]. With the ultimate goal of improving advanced NSCLC treatment outcomes in the first line setting, this thesis project presents a synthetic lethality discovery approach to identify CDDP-potentiating gene targets and, subsequently, describes validation studies on selected gene targets. The project was developed based on the following hypothesis: Chemotherapy-naive lung cancer cells exposed to sub-lethal concentrations of cisplatin will develop responses that contribute to cancer cell survival, disease progression, and development of treatment resistance. Identifying and blocking these responses will increase the potency of cisplatin against advanced NSCLC. As the project progressed, a lead target was identified and a more focused hypothesis was generated: Inhibition of PAPSS1 (3’-phosphoadenosine 5’-phosphosulfate synthase 1) will enhance the activity of cisplatin in the treatment of NSCLC.

The specific aims are as follows:

1) Select targets that enhance cisplatin activity in NSCLC cells based on a genome-wide siRNA screen and an Affymetrix gene expression study.

2) Perform secondary in vitro validation of the selected gene targets and select lead target(s) for in vivo validation.
3) **In vivo** validation of the lead gene target using at least two different model systems.

To identify novel cisplatin activity enhancing gene targets, two studies were performed: 1) A genome-wide siRNA screen designed to identify non-essential genes that when silenced would enhance the activity of low-dose ($IC_{10}$: concentration that causes 10% cell death) cisplatin against a NSCLC chemo-naive cell line A549; and 2) An Affymetrix microarray study designed to identify the differentially expressed genes in A549 cells that were untreated or treated with low-dose ($IC_{10}$) cisplatin. The studies were analyzed separately and the results were overlaid and compared. These results along with the **in vitro** validation of some of the candidate genes and pathways are presented in Chapter 2 of this thesis. A kinase subset of the genome screen was analyzed separately which led to the identification of our lead gene target, PAPSS1 (3’-phosphoadenosine 5’-phosphosulfate synthase 1). PAPSS1 is localized in the nucleus and is responsible for synthesizing the biologically active substrate, PAPS (3’-Phosphoadenosine 5’-Phosphosulfate), for sulfonation reactions. The analysis leading to the identification of PAPSS1 and its **in vitro** validation are described in detail in Chapter 3 of this thesis. Chapter 4 validates PAPSS1 as a cisplatin-potentiating target using a variety of model systems including 3D spheroid cultures as well as zebrafish and murine xenograft models.

### 1.2 Lung Cancer

Lung cancer is the leading cause of cancer mortality worldwide, with patients suffering from a late stage of disease at the time of diagnosis and a lack of effective therapeutic strategies available to treat lung tumours [4]. Lung cancer comprises two main subtypes: Small Cell Lung Cancer (SCLC) and Non-Small Cell Lung Cancer (NSCLC), which correspond to ~20% and ~80% of cases, respectively [5]. Lung adenocarcinoma (LAC) is the most common type of NSCLC, responsible for ~40% of all lung cancer cases and, unlike other subtypes, is associated with both smokers and never smokers [5, 6]. Squamous Cell Carcinoma (SqCC) is the other major NSCLC subtype and, along with SCLC, is characterized by its development in the central airways and close association with smoking [5]. The different lung cancer subtypes develop from unique cells of origin, involve the deregulation of specific oncogenic pathways and have diverse responses to conventional
chemotherapies, demonstrating the importance of considering histology in the clinical management of this disease [7].

Recently, large-scale genomics studies have revealed the genetic changes driving the development of lung cancer subtypes. Activating mutations in EGFR and KRAS as well as translocations involving ALK and RET are common in LAC while SqCC contain frequent mutations in PIK3CA and amplification of FGFR1 [8, 9]. Meanwhile, SCLCs are characterized by the dual inactivation of the tumour suppressor genes RB and TP53 and, less frequently PTEN [10]. With this increasing understanding of lung cancer biology has come the advent of targeted therapies to combat this devastating disease. These therapies target mutated components of key cellular pathways on which tumours cells have become dependent on for survival, a phenomenon known as oncogene addiction [11]. For example, tyrosine kinase inhibitors (TKIs) targeting LACs driven by mutant EGFR or ALK rearrangements have been clinically successful, highlighting the potential of designing drugs to specifically target the molecular mechanisms driving cancer development, a concept often described as “personalized medicine” [11-13]. However, despite these encouraging developments, significant problems remain. First, the majority of LAC patients are not candidates for these therapies as they have tumours without mutations in targetable genes, owing either to the lack of an identified driver or mutation in drivers such as mutant KRAS for which the development of inhibitors have proven elusive. Second, all patients eventually develop resistance to treatment with these targeted agents, either through secondary mutation of the target gene or downstream activation of their signalling pathways that sustain tumour growth. Furthermore, although targeted therapies have been successfully employed in the treatment of LAC, advances have lagged in SCLC and SqCC. In SCLC, the causative genetic changes involve inactivation of tumour suppressor genes - which are notoriously difficult to exploit therapeutically - while in SqCC, FGFR1 inhibitors have demonstrated mixed success, likely due to additional genetic determinants regulating response or the presence of alterative oncogene targets attributed to the amplified chromosome region [14]. Thus, while undoubtedly a major advancement in improving lung cancer patient outcomes, the traditional targeted therapy approach has failed to achieve the major goal of increasing long-term survival rates and new strategies to treat lung cancers are urgently needed.
1.3 The Concept of Synthetic Lethality

Synthetic lethality is traditionally defined as a condition where simultaneous mutation in two genes – but not either alone - leads to cell death [15, 16]. Where mutation in both genes impairs cellular fitness but does not cause lethality, this is described as a synthetic sick interaction [15-17]. Calvin Bridges first described synthetic lethality in 1922 when he observed that combinations of mutations in fruit flies lead to lethality (Figure 1.1) [17-19]. The term itself was later coined by Theodore Dobzhansky who made the same observations in 1946 [20]. For decades, synthetic lethal interactions were studied mainly in fruit flies; however, starting in the 1980s the search for synthetic lethal interactions expanded to other model systems including algae, yeast, and the nematode C. elegans [21-24]. These studies contributed significantly to our understanding of gene function, biological pathways, and genetic robustness, and identified many interactions potentially important in human cancer.

Figure 1.1. Synthetic Lethality: History and Evolution. The timeline indicates the major events that took place over the last century, from the first description of synthetic lethality to the recent development of technologies for high-throughput discoveries of synthetic lethal interactions.

The initial concept of screening for drugs that can specifically kill cancer cells carrying defined genetic changes was originally conceived using yeast as a model system. This basic approach combined with improvements in screening platforms subsequently allowed
chemical compound libraries to be assessed in human cancer cell lines, the first foray into exploring synthetic lethal targets for cancer causing genetic alterations [25]. However, chemical libraries suffer from difficulties in target identification, especially for larger libraries of diverse compounds, limiting their effectiveness in defining new synthetic lethal interactions on a global scale. Hypothesis-based assessment can alleviate these problems, as demonstrated by the successful validation of BRCA-deficiency and PARP inhibitor sensitivity in breast cancer [26]. However, the advent and rapid development of RNAi technology in the early 2000s allowed the first high-throughput genetic screens to be performed in human cancer cells driven by specific oncogenic mutations [27-29]. Consisting of well-based screens using transfection of individual siRNAs or pooled dropout/enrichment screens employing transduction of lentiviral shRNA libraries, this approach has proven invaluable for identifying synthetic lethal interactions for various oncogenes/tumour suppressor genes as well as chemosensitizing genes in lung and other cancers. Recently, major advancements in the generation of RNAi libraries, sequencing, high throughput screening platforms, and the recent development of CRISPR technology have further expanded the capacity to uncover synthetic lethal interactions in cancer[30-33]. In addition, contemporary screens are now relying more on computational and bioinformatics approaches such as statistically inferring synthetic lethal interaction pairs from cancer genomic data [34] These synthetic lethal screening approaches have been exhaustively reviewed recently [35-38].

While synthetic lethality can occur at the cellular or organismal level depending on the model being used, the goal in cancer treatment is to specifically eliminate a population of malignant cells, which is heterogeneous in nature [39]. With that perspective, drug combinations are designed with the ultimate goal of curing the disease, but more frequently, significant improvements in treatment outcomes are achieved through synergy, which is equivalent to a synthetic sick effect at the cell population level in the context of cancer biology. Hence, the concept of synthetic lethality encompasses a wide range of interactions involving genetic variations as a result of single-gene mutations, chromosomal translocation and deletions, as well as cellular responses to different cytotoxic and targeting pharmaceuticals. The various types of synthetic lethal/sick interactions from an oncology viewpoint are discussed below.
1.4 **Types of Synthetic Lethal Interactions and Identified Interactions in Lung Cancer**

The transformation of normal cells to cancer cells involves a step-wise evolution: a progressive series of genetic mutations that allow cells to acquire the hallmarks of cancer over time and become malignant [40]. These genetic changes cause deficiencies in, or addiction to, certain cellular processes and biological pathways that initiate transformation and are thus, prime targets for therapeutic intervention. However, cancer cells often develop resistance to broad spectrum and targeted treatments through cytoprotective responses and “back-up” proteins and pathways, respectively. This genetic robustness is another factor that can be targeted when using synthetic lethal strategies [16]. Here, we describe the different types of synthetic lethal interactions in two broad categories: 1) interactions that are purely based on genetic mutations and, 2) interactions that involve existing cytotoxic agents that are known to be effective in cancer patients (Figure 1.2). Furthermore, we highlight specific examples of synthetic lethality identified in lung cancer to date that may provide new strategies for therapeutic intervention (Table 1.1).
Figure 1.2. Types of Synthetic Lethal Interactions in the Context of Cancer. The various types of synthetic lethal interactions can be grouped into two categories: genetic-based and chemical-based interactions. Genetic synthetic lethality is primarily based on cancer-specific genetic alterations (blue normal cells undergo genetic changes that result in transformation to red cancer cells) which become susceptible to further induced changes in gene expression resulting in synthetic lethality. Chemical synthetic lethality describes synthetic lethal interactions between inherent or induced genetic alterations and broad-spectrum therapeutics (chemosensitization) as well as synergistic outcomes from the use of two or more chemotherapeutics. Please see Sections 1.3.1 and 1.3.2 for full description of each type of interaction. (LOF = loss-of-function, GOF = gain-of-function, blue cell = normal cell, red cell = cancer cell, grey cell = dead cancer cell, $A_1$ = passenger mutation of isoform 1, $A_2$ = isoform 2 of passenger gene)
<table>
<thead>
<tr>
<th>Interactor 1</th>
<th>Interactor 2</th>
<th>Type of Interaction</th>
<th>Method of Discovery</th>
<th>Lung Cancer Subtype</th>
<th>Year discovered</th>
<th>PMID</th>
</tr>
</thead>
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<tr>
<td>PAPSS1</td>
<td>Cisplatin (or other DNA damaging agents)</td>
<td>Cisplatin</td>
<td>Chemo-sensitization</td>
<td>RNAi Screen (siRNA) + Low-Dose Cisplatin</td>
<td>LAC</td>
<td>2015</td>
</tr>
<tr>
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<td>Cisplatin</td>
<td>Cisplatin</td>
<td>Chemo-sensitization</td>
<td>RNAi Screen (siRNA) + Cisplatin</td>
<td>LAC</td>
<td>2014</td>
</tr>
<tr>
<td>dUTPase</td>
<td>FUDr/pemetrex ed</td>
<td>Cisplatin</td>
<td>Chemo-sensitization</td>
<td>Hypothesis Based</td>
<td>LAC; bronchioalveolar carcinoma; LCC</td>
<td>2012</td>
</tr>
<tr>
<td>mKRAS</td>
<td>PKCι Aggregation (via Oncrasin-1 treatment)</td>
<td>GOF + GOF</td>
<td>Chemical library</td>
<td>Compound Screen</td>
<td>LAC</td>
<td>2008</td>
</tr>
<tr>
<td>mKRAS</td>
<td>mEGFR</td>
<td>GOF + GOF</td>
<td>Hypothesis Based</td>
<td>Computational - EGFR Interactome Mapping in Lung Cancer Genomic Data</td>
<td>LAC</td>
<td>2015</td>
</tr>
<tr>
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<td>mBRAF</td>
<td>GOF + GOF</td>
<td>Hypothesis Based</td>
<td>Chemical library</td>
<td>LAC</td>
<td>2016</td>
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<tr>
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<td>ARHG5</td>
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<td>GOF + LOF</td>
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<tr>
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<tr>
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<td>GOF + LOF + LOF</td>
<td>RNAi Screen (shRNA) + Gefitinib</td>
<td>LAC</td>
<td>2014</td>
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<tr>
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<td>NF-kB + EGFR-i</td>
<td>GOF + LOF + LOF</td>
<td>RNAi Screen (shRNA/siRNA) + Gefitinib</td>
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<td>GOF + LOF + LOF</td>
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<tr>
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<td>LOF + LOF</td>
<td>Hypothesis Based</td>
<td>LAC</td>
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<td>Tankyrase 1</td>
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<td>BRG1</td>
<td>LOF + LOF</td>
<td>Hypothesis Based</td>
<td>Computational - Global gene expression analysis; cancer databases</td>
<td>SCLC</td>
<td>2014</td>
</tr>
<tr>
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<td>CDKN2A</td>
<td>LOF + LOF</td>
<td>Proteome/transcriptome profiling</td>
<td>Compound library screen</td>
<td>LAC; SCLC</td>
<td>2016</td>
</tr>
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<td>phenformin</td>
<td>LOF + LOF</td>
<td>RNAi Screen (shRNA) + Gefitinib</td>
<td>LAC</td>
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<td>Hypothesis Based</td>
<td>LAC; LCC</td>
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<td>Sensitization</td>
<td>RNAi Screen (shRNA)</td>
<td>SCLC</td>
<td>2002</td>
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</table>

**GOF** = gain-of-function; **LOF** = loss-of-function; **LAC** = lung adenocarcinoma; **SCLC** = small-cell lung cancer; **LCC** = large cell carcinoma; **m-(gene)** = mutant variant of the gene; **(gene)-i** = inhibitor of gene product
1.4.1 Genetics-Based Synthetic Lethality

As mentioned above, tumour cells acquire mutations that allow them to grow rapidly over time. While these characteristics give cancerous cells advantages in proliferation and survival, they also become therapeutic targets from a treatment perspective. The differential regulation of genes between cancer cells and their corresponding normal cells allow researchers to identify those that can be targeted to induce synthetic lethality in a cancer-specific manner. The various types of interactions are described below.

Loss-of-Function & Loss-of Function

Loss-of-function (LOF) mutations in tumour suppressors are extremely common in human cancers. One such example is the genome guardian p53, the most frequently mutated gene in human cancers, ranging from 25-50% in various tumour types including ovarian, breast, colorectal, head and neck, and lung cancers [41]. Although p53 mutations have been studied extensively, it has been a difficult target as it has no enzymatic activity and primarily functions through protein-protein interactions [42]. In attempt to target p53, efforts have been made to restore the wild-type function of this “undruggable” target [43]. Another therapeutic approach is to exploit the vulnerabilities or genetic dependencies arising from loss of wild-type p53 functions [44]. For example, based on differential gene expression analyses, Wang and Simon proposed a list of 98 candidate genes that, when suppressed, may induce synthetic lethality in p53-deficient cancers [45]. We define such a strategy as LOF/LOF as it involves inhibiting a gene/pathway/function in the background of genetic or pharmacological inactivation of another gene to induce synthetic lethality.

To date, the biggest clinical success in synthetic lethal targeting is the use of PARP (poly(ADP-ribose) polymerase) inhibitors against tumours with BRCA mutations. BRCA1 and BRCA2 deficiencies, either of which causes defects in homologous recombination (HR), have a significant role in hereditary breast and ovarian cancers [46]. It is known that mice lacking PARP expression are viable but are defective in repairing single-stranded DNA breaks. Loss of PARP function results in the dependence on the use of HR to repair DNA damage [47]. Based on these observations, Bryant et al. hypothesized and then demonstrated that BRCA-deficient cancers are hypersensitive to PARP inhibitors [47]. The discovery of this truly synthetic lethal interaction (by definition) has revolutionized our
approach to the treatment of BRCA-associated cancers and served as the paradigm for uncovering synthetic lethal interactions.

*PTEN*, the second most frequently mutated tumour suppressor in cancer, is a similar therapeutic target [48]. Inspired by the synthetic lethality induced by loss of BRCA2 and PARP functions, Mendes-Pereira *et al.* demonstrated that cancers with PTEN deficiency, which also causes HR defects, are sensitive to PARP inhibitors [49]. This synthetic lethal interaction was validated *in vitro* and *in vivo*. As a result, PARP inhibitors are currently in Phase II clinical trials for treatment of PTEN-deficient cancers. Exploiting deficiencies in DNA repair proteins has since revealed additional synthetic lethal interactions with particular relevance to lung cancer. Similar to the case of PARP and BRCA, Riabinska *et al.* observed that cancers with ATM-deficiency are defective in HR and dependent on nonhomologous end joining (NHEJ) for DNA repair and cell survival [50]. Through genetic and pharmacological methods, they subsequently demonstrated that inhibition of an essential NHEJ protein PRKDC induces synthetic lethality specifically in ATM-deficient cancers, but not normal cells or cancer cells with active ATM. Likewise, after developing a potent and selective inhibitor of the DNA damage response (DDR) kinase ATR, Reaper and colleagues found that it induced synthetic lethality specifically in ATM- or p53-deficient cancers in the context of genotoxic stress [51]. Since ATM is mutated and inactivated in ~7% of LACs, these findings provide a potential therapeutic strategy for a large subset of patients, one which is especially resistant to standard chemotherapeutics [52].

While the above examples were mainly identified in other cancer types, many of the described genes are also disrupted in lung cancer suggesting that similar synthetic lethal interactions may also exist in this context. However, screens for lung cancer specific LOF/LOF synthetic lethal interactions have also been performed. For example, through a chemical library screen, Shackelford and colleagues found that LKB1 (STK11) deficient lung cancer cell lines were acutely sensitive to phenformin treatment, which inhibits mitochondrial function [53]. LKB1 is mutated in ~20% of NSCLC and this work, subsequently validated in LKB1 mutant mouse models, suggests that metabolism based therapeutics may be effective in this subset of cancer patients. Using a LOF whole-genome shRNA screen, inhibition of the canonical Wnt pathway, specifically the positive regulators tankyrase 1 and 2, was found to induce cell death in LAC cells only in the context of EGFR
inhibition [54]. Furthermore, computational approaches have revealed LOF/LOF synthetic lethality of MAX and BRG1 in SCLC and RB1 and CDKN2A in both SCLC and NSCLC [55, 56].

Lastly, it is important to note a unique subcategory of LOF/LOF interactions that only occur in the presence of a gain-of-function (GOF) oncogene mutation. These interactions typically involve inhibiting the mutant oncogene with a small molecule inhibitor and another gene with pharmacological or genetic methods. Since the inhibitors only work – and hence synthetic lethality is only induced - in the context of the GOF mutation, we term this association GOF/LOF/LOF, even though only two genes are involved. Examples of this association include the use of EGFR kinase inhibitors in the background of mutant EGFR (mEGFR). For instance, using RNAi screening in the presence of the EGFR kinase inhibitor Erlotinib, Bivona and colleagues found that inhibiting FAS and other components of the NF-κB pathway specifically enhanced cell death in mEGFR LAC cell lines [57, 58]. Likewise, Sudo et al. found a similar association between NF-κB inhibition and Gefitinib (another EGFR kinase inhibitor) and also identified a novel gene candidate, PRKCSH, which induced cell death in Gefitinib-treated mEGFR LAC cells [59]. Finally, a pooled shRNA screen in KRAS mutant cancer cells - which are dependent on MEK activation - found that inhibition of BCL-XL cooperated with MEK inhibitors to induce cell death[60]. These findings were validated using the MEK inhibitor selumetinib and the BCL-XL inhibitor ABT-263 in a genetically engineered KRAS-driven mouse model of lung cancer, highlighting the potential clinical relevance of this interaction.

*Gain-of-Function & Loss-of-Function (Synthetic Dosage Lethality)*

While loss of tumour suppressor functions is a common characteristic in cancer cells, activation of various oncogenes through gain-of-function (GOF) mutations is another major contributor to tumour development. Like LOF mutations, GOF mutations rewire cancer cells, making them susceptible to additional changes in cellular functions that would cause little or no harm to normal cells. When lethality occurs as a result of one gene being genetically activated (GOF) and another being inactivated through genetics or drug targeting (LOF), the interaction is known as synthetic dosage lethality.

As an example, constitutive activation of RAS signaling, particularly KRAS, is known to be the oncogenic driver for approximately 20% of all human cancers [61]. Cancers of the
pancreas, colon, and lung are known to have high frequencies of KRAS mutations [62]. KRAS has long been considered an “undruggable” target due to the lack of suitable binding pockets for small molecule inhibitors [63]. To target KRAS mutants, several groups have utilized high throughput RNAi screening to identify synthetic lethal partners of activated mutant KRAS. For instance, Luo et al. performed a genome-wide shRNA screen in colorectal cancer cells and found that KRAS mutants are hypersensitive to PLK1, APC/C, and proteasome inhibition relative to isogenic cells with wild-type KRAS [64]. Other synthetic lethal partners that have been identified through RNAi screening within an activated KRAS background include STK33, TBK1, WT1, CDK1, CDK4, GATA2, and Snail2, most of which were conducted in colon and lung cancer cells[65-71] and have been reviewed elsewhere.

Hyperactivation of members of the MYC protein family is very common in a wide range of cancers. For instance, c-MYC is amplified in about 10% of LAC and c-MYC, MYCL or MYCN amplification/overexpression occurs in >20% of SCLC. MYCs are transcription factors - and thus, difficult to inhibit directly – thus, synthetic lethal strategies are being used to define novel treatments for cancers driven by the activation of these proteins. Inhibition of mTOR, Aurora A/B, SAE2, or CDK1 has been shown to induce synthetic lethality in MYC-driven cancers [72-75]. Synthetic lethal relationships between hyperactivated MYCN and suppression of BRD4 and CSNK1e have also been demonstrated in acute myeloid leukaemia and neuroblastoma, respectively [76, 77]. Inhibitors targeting CDK1 and BRD4 are now in Phase II clinical trials against MYC-amplified tumours. Recently, through a pooled shRNA screen, inhibition of PRKDC was found to specifically induce synthetic lethality in SCLC cell lines with amplification/overexpression of MYC family members but not those without [78]. This was attributed to the role of PRKDC in controlling MYC protein levels as well as facilitating repair of MYC-induced DNA damage. These results could be recapitulated with a PRKDC inhibitor, NU-7441, suggesting a possible therapeutic avenue for this aggressive lung cancer subtype.

Lastly, although EGFR kinase inhibitors have proven effective at improving outcomes of LAC patients with activating EGFR mutations, all patients treated with these inhibitors eventually develop resistance and relapse. Identifying the key factors necessary for mEGFR-mediated tumorigenesis may offer avenues for combination based therapies that can overcome EGFR kinase inhibitor resistance. Through characterizing the LAC-specific
mEGFR interactome through global analysis of protein–protein interactions and phosphorylation, Li and colleagues identified 8 key proteins necessary for survival mEGFR lung cancer cell lines in addition to EGFR itself: GRB2, MK12, SHC1, ARAF, CD11B, ARHG5, GLU2B, and CD11A [79]. When inhibited through siRNAs, these genes induced cell death specifically in mEGFR, but not EGFR wild-type, LAC cells suggestive of a synthetic lethal relationship between these events. Indeed, ablation of ARHG5 in mEGFR LAC cells induced apoptosis and was shown to interact with downstream EGFR signalling proteins including SHC1 and GRB2, highlighting its potential as a target for combination based therapy.

**Gain-of-Function & Gain-of-Function**

With the tools currently available, it is relatively easy to identify synthetic lethal interactions using gene knockdown or knockout approaches. In contrast, there has yet to be a large-scale GOF screen in human cells to search for synthetic lethality in cancers driven by known oncogenes. It is, however, not impossible to define synthetic lethal interactions that are based on two gain-of-function mutations using information derived from human tumour evolution. For instance, it has been known for years that oncogenic mutations in KRAS and EGFR are not only common, but also mutually exclusive in LAC [80-82]. It was typically assumed that since the two oncogenes work through the activation of similar pathways, mutation in both genes was functionally redundant and thus, not positively selected during tumour development. However, in a recent study, Unni et al. discovered that these two mutations are mutually exclusive because activation of both genes in lung cells induces synthetic lethality, which is selected against during tumour evolution [83]. Expressing both mutant oncogenes in a mouse model of lung cancer led to the selection of tumours expressing a single oncogene while forced expression of mEGFR or mKRAS in LAC cells with endogenous mutations in the reciprocal oncogene induced cell death through uncontrolled macropinocytosis and catastrophic cell vacuolization, likely through increased MAPK signalling. From a clinical perspective, while it is challenging to increase expression of a gene product, this study suggests that there may be opportunities to activate combinations of pathways to induce lethality. The method through which this interaction was revealed and validated suggests that additional synthetic lethal interactions may be identified through exploring co-activation of mutually exclusive driver mutations and their respective pathways. Indeed, a subsequent study revealed a similar
association between mutant BRAF and mKRAS in LAC, demonstrating the validity of this approach.

Collateral Lethality

A more recent concept of synthetic lethality is “collateral lethality” [84]. In the process of malignant transformation, some tumour suppressor genes may be inactivated through chromosomal deletions. While loss of heterozygosity or homozygous deletions results in complete inactivation of a tumour suppressor - which in itself enables cancer-specific targeting of synthetic lethal partners as described above - collateral lethality takes advantage of “passenger” or “neighboring” genes that are co-deleted “unintentionally” in this process [84, 85]. These passenger genes may encode for housekeeping functions that are essential to cell viability but are masked by the presence of redundant genes encoded elsewhere to complement for the loss. Targeting homologues of these passengers forms the basis of collateral lethality. As an example, ENO1 is a gene that is homozygously deleted in glioblastoma (GBM) as a result of deletion in the 1p36 tumour-suppressor locus [85]. This gene encodes for enolase, an enzyme that is essential for glycolysis. Although ENO1 accounts for up to 90% of the enolase activity in GBM, its loss is tolerated through the expression of ENO2 which is exclusively expressed in neural cells [62]. Loss of ENO2 does not cause reduced viability in the presence of ENO1 expression, but GBM cells with deletion in 1p36 are highly sensitive to ENO2 inhibition due to collateral lethality [85]. Muller et al. have identified other homozygously deleted essential genes that have potential collateral lethal partners in GBM [85].

To date, the only described instance of collateral lethality in lung cancer involves SMARCA4 - a chromatin remodelling helicase - and its parologue, SMARCA2. Unlike the ENO1/ENO2 example, SMARCA4 is a true tumour suppressor gene and is directly inactivated through mutations or deletions in approximately 10-15% of LACs. However, unlike other tumour suppressors, SMARCA4 - along with SMARCA2 - performs cellular housekeeping functions rather than preventing abnormal cell growth. Thus, inhibition of SMARCA2 in the background of SMARCA4 genetic inactivation leads to synthetic lethality as the cell loses the ability to complete an essential function necessary for survival, as is the case with
EN01/ENO2 in GBM. Inhibitors targeting SMARCA2 may therefore prove effective in the large subset of LAC patients with SMARCA4 mutations [86-89].

1.4.2 Synthetic Lethal Interactions Involving Broad-Spectrum Pharmaceuticals

As introduced earlier, the traditional definition of synthetic lethality/sickness involves defects (hyperactivation or inactivation) in a pair of genes. In the context of cancer, however, synthetic lethal/sick interactions also apply to gene-drug and drug-drug interactions. To date, cancer treatment relies heavily on the use of broad-spectrum therapeutics that target essential cellular processes such as DNA replication and mitosis: processes that are particularly important to rapidly dividing cells. Although these cytotoxic agents are highly effective, their uses have been limited by narrow therapeutic windows, which result in severe and potentially life-threatening toxicities, and the development of resistance over time. Nonetheless, these agents have provided significant therapeutic benefits to patients (especially in the context of intratumoural heterogeneity) and cannot be easily supplanted as standard of care for many cancers. With a better understanding of cancer genetics and the mechanisms of drug action, a tremendous amount of effort is being placed on improving the efficacy of existing cytotoxic agents through synthetic lethal approaches, whether through selecting the proper drug based on tumour-specific defects (chemosensitization) or developing drug combinations to achieve synergy (synthetic sickness).

Gene-Drug Interactions

Cisplatin (CDDP) is undoubtedly one of the most successful chemotherapeutics ever discovered for cancer treatments. While its use and clinical success is widespread, it causes severe side-effects including nephrotoxicity, neurotoxicity, and ototoxicity [90]. Furthermore, resistance to platinum-based drugs is common. To overcome these challenges, many groups have attempted to develop combination products comprising CDDP and targeted treatments by performing high-throughput RNAi synthetic lethal screens to better understand the mechanisms of resistance and the genetics underlying the sensitivity of cancer cells to CDDP and other DNA damaging agents. For instance, cancers with defects in DNA damage repair such as BRCA mutations are hypersensitive to DNA
cross-linkers including CDDP, carboplatin, and mitomycin C [91]. Other genes that have been identified as potential therapeutic targets for sensitization to CDDP treatment include AMBRA, and PRKAB1 [92, 93]. In fact, numerous genes have been identified as chemosensitizing targets where gene knockdown or target inhibition via small molecules sensitizes tumours to multiple chemotherapeutics. One such target is CABYR, a cancer testis antigen in lung cancer that sensitizes NSCLC to paclitaxel and CDDP [94, 95]. Inhibition of components of the ATR-CHK1 checkpoint signaling pathway appears to sensitize ovarian cancer cells to multiple DNA damaging agents [92, 96]. PAPSS1, one target that our group identified (please see Chapter 3), is a nuclear enzyme that produces the obligate substrate for sulfonation reactions which when inhibited, sensitizes NSCLC cells to a wide range of DNA damaging agents [97]. Swanton et al. have also identified CERT, a ceramide-binding protein, as a target that enhances the activity of CDDP and paclitaxel in lung cancer cells, paclitaxel and 5-Fu in colorectal carcinoma cells, and paclitaxel and doxorubicin in breast cancer cells[98]. To achieve maximal therapeutic effects, multiple targeting may be necessary. This is exemplified by De et al., who demonstrated that the best treatment outcome was achieved in vivo when triple negative breast cancer was treated with carboplatin in combination with both an mTOR and a PARP inhibitor [99]. Similar to these studies on chemotherapeutics, groups have also aimed to identify genes that modify the sensitivity of lung cancer cells to radiation, which is commonly used in the treatment of lung cancer patients. For example, a whole genome RNAi screen found that inhibition of proteasome subunits including PSMA1, worked synergistically with ionizing radiation to induce killing of lung cancer cells[100].

Drug-Drug Interactions

Traditionally, drug combinations have been defined by simply combining two drugs that display the best single agent activity with non-overlapping toxicities for a specific cancer indication. Often, two drugs of different mechanism of actions are also chosen as a broad-spectrum therapeutic strategy. These approaches have led to numerous successes as well as failures. It is now apparent that some drug combinations work synergistically through synthetic lethality while others result in antagonism, where the combinatorial effects turn out to be worse than the single agents. As an example, the antimetabolite gemcitabine and the topoisomerase I inhibitor topotecan have been shown to be antagonistic in ovarian
cancer cells [101]. Peters et al. found that the combination of CDDP and gemcitabine is synergistic \textit{in vitro} and \textit{in vivo} using ovarian, head and neck, and colorectal cancer models [102]. This drug combination is currently used for the treatment for at least seven different cancers types including ovarian and NSCLC. Likewise, irinotecan plus CDDP was demonstrated to provide a synergistic benefit in the treatment of metastatic SCLC as compared to etoposide plus CDDP, which was the standard of care [103]. For \textit{in vitro} and \textit{in vivo} studies, calculation of combination indices using the Chou-Talalay method has been the standard method for determining combination effects [104, 105]. To determine the clinical effects of drug combinations, Kang et al. analyzed over 1000 Phase II clinical trials and determined the clinical synergy and antagonism of different doublets based on overall response rates [106].
2. Combined Use of Gene Expression Modeling and siRNA Screen Identifies Genes and Pathways Which Enhance the Activity of Cisplatin When Added at No Effect Levels to Non-Small Cell Lung Cancer Cells in *vitro*

2.1 Introduction

Future approaches to increase the survival of patients with aggressive cancers must address the problem of tumor heterogeneity by remaining focused on broad-spectrum drugs which already provide some meaningful therapeutic benefits. Standard-of-care drugs (e.g., cisplatin, doxorubicin, irinotecan, gemcitabine) will not be replaced in the near future because when used in combinations they produce significant improvements in overall survival \[107-111\]. These therapeutic benefits, however, are typically achieved when using drug doses that cause acute and chronic toxicities. The work presented in this chapter is attempting to define strategies that will enhance the activity of these drugs and reduce their toxicities through approaches that consider how cancer cells protect themselves from the cytotoxic effects of the drugs. In cases where drug delivery is limited by the inadequate blood supply through tumor associated blood vessels as well as tissue specific barriers (e.g., blood-to-brain or stromal barriers), it is recognized that tumor cells are exposed to a gradient of drug concentrations \[112\]. Some regions within the tumor are exposed to lethal concentrations while others are exposed to sub-lethal levels of the drug(s). Tumor cells exposed to sub-lethal doses develop survival responses that protect them while also allowing for selection of drug-resistant tumor cell subpopulations. The ability of cancer cells to adapt via intrinsic and acquired cytoprotective responses when first exposed to sub-lethal drug concentrations is one factor that limits the effectiveness of chemotherapeutic drugs.

Here, we describe studies to better understand how a chemotherapy-naive non-small cell lung cancer (NSCLC) cell line responds when exposed to a cisplatin (CDDP) dose that causes less than a 10% loss in cell viability (IC\(_{10}\)) as determined in a 3-day high content
screening assay. This drug dose was defined as the no-observed-effect level (NOEL). Two studies were completed and the results were combined to develop an understanding of how tumor cells respond when exposed to sub-lethal CDDP doses and to determine whether these responses could be exploited to enhance CDDP activity (i.e. causing a NOEL of CDDP to become lethal). A microarray study was performed to examine changes in gene expressions in an adenocarcinoma NSCLC cell line A549 following treatment with a NOEL of CDDP. A genome-wide siRNA screen was completed in parallel to identify genes that could be targeted to enhance the cytotoxic effects of CDDP in these cells. The goal of this study is not only to identify genes and pathways that are over-expressed in response to a NOEL of CDDP, but also to identify the genes and pathways within this list that when silenced, transform the NOEL of CDDP to a lethal dose. When analyzing the two datasets, we did identify targets from the siRNA screen that could potentiate CDDP activity but were not differentially expressed in the microarray study. Some of those targets were further explored and the results are disclosed in Chapter 3 [97]. In this chapter, we linked results highlighting genes that were overexpressed following low-dose CDDP exposure to genes that when silenced enhanced the cytotoxic effects of low-dose CDDP. Four gene candidates (RRM2B, CACYB, ALDH3A1, and FHL2) were identified as potential CDDP modulators. Further, the double strand DNA homologous repair and INO80 chromatin remodeling pathways were recognized as important targets for improving the effectiveness of low-dose CDDP.

2.2 Methods

2.2.1 Study Design

A schematic diagram of the study design is shown in Figure 2.1. Briefly, a high-throughput siRNA screen (see below for detailed methods) was performed to identify genes and pathways that could be inhibited to enhance the cytotoxic effects of low-dose CDDP against A549 cells. A gene expression study was performed separately to identify differentially expressed genes and pathways in response to the same NOEL of CDDP. The two independent studies were then compared at the gene and pathway levels.
Figure 2.1. Experimental design. The schematic diagram of the siRNA screen is illustrated on the left and the microarray gene expression study on the right. The data from the two studies were analyzed as described in the methods section. The top hits identified from the siRNA screen the differentially expressed genes were compared at the genetic level to identify genes that may be targeted to enhance CDDP activity. The two lists were separately processed through Pathway Studio and the resulting pathways were compared subsequently to determine the most critical pathways to be targeted in combination with CDDP use.
2.2.2 Cell Culture

A549 and H460 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained at 37°C and 5% CO₂ in RPMI 1640 supplemented with L-glutamine (Gibco) and 10% fetal bovine serum (Gibco).

2.2.3 Defining the No-Observed-Effect Level of Cisplatin

The IC₁₀ of CDDP in a 3-day high content screen was defined in these studies as the no-observed-effect level (NOEL). To establish the IC₁₀ of CDDP, cells were seeded at 2000 cells/well in a flat-bottom 384-well plate (Greiner Bio-One). CDDP dose response curves were determined by adding CDDP (Mayne Pharma; ready-to-inject stock solution diluted with PBS) at various concentrations to the cells. At 72 hours post-treatment, the cells were stained for 20 minutes with dye exclusion marker Hoechst 33342 (16.2 µM, Invitrogen) and the nuclear stain ethidium homodimer I (EthD-I; 1µM, Biotium) and then imaged via the IN Cell Analyzer 1000 (GE Healthcare). Nine non-overlapping images were taken per well and analyzed using the IN Cell Developer Toolbox v1.9 software (GE Healthcare). Viable cell counts were determined by subtracting dead cell counts (EthD-I stained) from the total nuclei count (Hoechst-stained) in each well. The raw data were then normalized to untreated controls to generate a dose response curve, where a fraction affected (Fa) of 0 represents null effect from treatment and a Fa of 1 indicates 100% cell death. The dose that induced a Fa of 0.1 was the IC₁₀. At this concentration, no morphological changes were observed relative to untreated controls, hence it was chosen as the NOEL for subsequent studies.

2.2.4 Whole Genome siRNA (WGS) Screen

A549 cells were seeded at 2000 cells/well in 384-well plates (Greiner Bio-One). Cells were transfected with the human siGENOME library (Dharmacon) with each well containing a SMARTpool of 4 siRNA duplexes targeting one of the 21,121 genes within the human genome. Transfection was performed 24 hours after plating using the lipid reagent RNAiMAX (Life Technologies). To ensure minimal toxicity from the transfection reagent and maximal gene silencing, the amount of RNAiMAX that caused less than 10% cell death when complexed with a non-targeting siRNA (0.065 µL/well) and the siRNA concentration

21
(25 nM) that consistently caused the most A549 cell death with polo-like kinase 1 (PLK1) knockdown, were selected for the screen. At 24 hours post-transfection, CDDP was added to achieve a final concentration of either 0 or 0.551 µM (the IC_{10} against A549 cells under the screening conditions) in triplicates. The cells were fixed with 95% ethanol and the nuclei were stained with 16.2 µM of Hoechst 33342 (Life Technologies) at 72 hours following CDDP treatment. The plates were then imaged using the IN Cell Analyzer 1000 (GE Healthcare), an automated fluorescent microscopy platform that enables high content screening. Cell counts were determined via the IN Cell Developer Toolbox software. All siRNA dilutions were carried out using a Microlab STARlet (Hamilton Robotics). Cell seeding, siRNA addition to cells, CDDP treatment, ethanol fixation, and nuclear staining procedures were performed via Hydra DT (Thermo Fisher Scientific). The Multidrop Combi Reagent Dispenser (Thermo Fisher Scientific) was also used to facilitate the ethanol fixation process. The entire WGS was completed in 18 cycles, with each cycle being an iteration of a 6-day experiment.

To validate the screening conditions, a preliminary kinase screen (PKS) was performed with a subset of the siGENOME library targeting 640 protein kinases using the protocol described above. The whole genome screen (WGS) was subsequently completed. Each plate had four controls: RNAiMAX lipid control, PLK1, scramble (non-targeting siRNA), and BRCA2 (siRNA targeting breast cancer type 2 susceptibility protein). PLK1 served as a transfection efficiency control [113] while BRCA2, the silencing of which is a known CDDP potentiator, was a positive control [114]. These controls were randomly spotted in the first four columns of each plate to account for positioning effects. For quality control, full plates of controls with no siRNA, +/- lipid, and +/- CDDP were screened in triplicates once a week. A CDDP dose response curve was also generated on each CDDP treatment day of the screen to ensure accurate drug dilution and addition.

### 2.2.5 siRNA Screen Analysis

Cell counts were determined by estimating the number of stained cells in each image using our cell detection algorithms [115] and the median cell counts were used to compare cell survival in untreated versus CDDP-treated cells when a particular gene was silenced. The median was chosen over the average to account for single-well experimental failures (e.g.
transfection failures) by using the values of the other two wells. A Gene Score was computed for each gene using the equation: Gene Score = (100-|Survival Index – 100|) x Potentiation Effect. The Gene Score was then used to rank the list of genes based on each gene’s similarity to the positive BRCA2 control. The Survival Index is an estimate of the lethality of gene knockdown alone and is determined based on the no-CDDP median cell count normalized to the median cell counts from RNAiMAX transfection reagent controls. The Potentiation Effect is an estimate of the extent of CDDP potentiation calculated by taking the Difference of the median cell counts in CDDP-treated wells from the corresponding untreated wells and then normalizing to the difference detected from the positive control BRCA2.

For the entire screen dataset, a linear mixed effects model was employed to statistically account for the differences in response due to the well location of the plate (e.g. plate edge effects where lower cell counts are typically observed and reagent pipetting effects where a fixed pattern of reagent dispensing is noticed) and plate-to-plate experimental difference. Multiple comparison adjustments were performed using the Benjamini-Hochberg approach for p-values [116]. All hits presented here were considered statistically significant based on the adjusted p-values. For pathway analysis, the distributions of the Difference, Survival Index, and Gene Score values were assessed for threshold variability and a high-confidence set of non-lethal gene knockdown candidates was defined (90th percentile of Difference, 75th percentile of Survival Index), reducing the list of genes from 21121 to 480. These genes were then assessed for pathway enrichment.

2.2.6 Microarray Analysis

Cells were seeded in 96-well plates at 5000 cells/well. On the following day, the cells were either untreated or treated with the empirically determined IC_{10} of CDDP. At 24 and 48 hours post-treatment, total RNA was isolated using the Qiagen RNeasy plus mini kit (Qiagen). The RNA was quantified using the ND-1000 UV-VIS spectrophotometer (NanoDrop Technologies) and RNA integrity was verified with the Agilent 2100 bioanalyzer (Agilent Technologies). Three independent experiments were performed and all RNA samples were sent to The Centre for Applied Genomics (TCAG) at the Hospital for Sick Children (Toronto, ON), where probe labelling and hybridization were performed.
according to manufacturer’s protocol (Affymetrix). The microarray analysis was performed using the Affymetrix GeneChip Human Exon 1.0 ST Array, which contains approximately 40 probes per gene, enabling expression analysis at both the exon level (which distinguishes between different isoforms) and at the gene level (where a single expression value summarizes data collected from all probes of the same gene). Probe cell intensity (CEL) files from TCGA were analyzed using the Affymetrix Expression Console software and Affymetrix Power Tools. For quality control, Array data were normalized using robust multi-array average (RMA) normalization [117]. Density and boxplot distributions of signal intensities were examined for consistency across all samples before and after normalization. Multidimensional scaling (MDS) was applied: one outlier (a sample set obtained at 24h) was identified and was discarded from the analysis. Differential and alternate gene expression analyses were carried out using the Limma package in R [118] to contrast treated samples (NOEL of CDDP) to control samples (no CDDP). A p-value cut-off of 0.005 was used to obtain a short-list of differentially expressed genes.

2.2.7 Identification of Cisplatin Enhancers from the Two Datasets

The siRNA screen parameters (Survival Index, Difference, and Gene Score) were matched for each gene within the list of differentially expressed genes from the microarray study. A Gene Score rank of <2000 (top 10%) was applied to narrow the list of differentially expressed genes from 151 to 10. Of these potential targets, those with favourable siRNA screen parameters suggestive of CDDP potentiation (Survival Index and % Viability in the presence of CDDP) were selected for further validation studies (see below). Fisher’s exact test was used to evaluate the statistical significance of the overlapped gene targets.

2.2.8 Pathway Analysis

All pathway analyses were completed using Pathway Studio (Ariadne Genomics) [119] and the bioinformatics resource DAVID [120, 121]. To compare the siRNA screen and the microarray data, the gene lists obtained from the two studies were analyzed separately for enriched cellular processes, cell signalling, receptor signalling, and metabolic pathways. Pathways with a p-value of <0.05 were considered significant and overlaid for comparison.
2.2.9 Target Validation Studies: Quantitative RT-PCR and Clonogenic Assays

For quantitative RT-PCR, total RNA was extracted as described above. The QuantiTect Reverse Transcription Kit (Qiagen) was used to eliminate genomic DNA and to synthesize cDNA from 1 µg of total RNA. RT-qPCR was performed in triplicates via the 7900HT system (Applied Biosystems, Foster City, CA). Reactions were prepared with the 2X TaqMan fast advanced master mix and 20X Taqman gene expression assays (Applied Biosystems) according to manufacturer's protocol. Data were analyzed using the SDS2.2 software (Applied Biosystems) and the relative messenger RNA quantity was determined using the ddCt method with GAPDH as the endogenous control. Statistical analysis was performed using multiple t-tests followed by Holm-Sidak multiple tests correction.

For colony formation assays, cells were seeded at 200,000 cells/well in 6-well plates and then transfected with a pool of three different siRNA Stealth duplexes (Life Technologies) targeting the gene of interest using RNAiMAX. The Stealth RNAi negative control kit (Life Technologies) was used as scramble control. At 24 hours post-transfection, the cells were exposed to an empirically determined IC$_{10}$ of CDDP for 24 hours, harvested by trypsinization, and then seeded at 500 cells per well in triplicates. The cells were incubated at 37°C, 5% CO$_2$ for 14 days without disturbance. The colonies formed were fixed with 6.25% glutaraldehyde (Sigma) and stained with 0.5% crystal violet (Sigma) for 30 minutes, washed with distilled water, dried overnight, and then counted the following day. Plating efficiency (PE) was defined as the percentage of trypan blue excluding cells that formed colonies of >50 cells (PE = [(no. of colonies formed/no. of cells seeded) x100%]). Statistical analyses were performed using two-way ANOVA followed by the Holm-Sidak test. An adjusted p-value of less than 0.05 was considered statistically significant.

2.2.10 Pathway Validation in Yeasts

Yeast strains were derived from the MATa, BY4741 Yeast Knockout Collection [122]. Overnight cultures were diluted to an optical density at 600 nanometers (OD600) of approximately 0.07 in media with or without 40µM CDDP (Sigma) and grown in a Tecan M200 plate reader for 24 hours at 30°C. Shaking and OD600 measurements were done
every 30 minutes during the growth period. Growth curves were analyzed essentially as described [123, 124]. Briefly, 4 to 6 replicate curves for each condition were generated and the area under each curve was expressed as a proportion of the corresponding wildtype (WT) untreated sample. Multiplying the fitness effect of CDDP on WT cells by the fitness effect of the indicated mutation created the Expected fitness based on the product or multiplicative model [125]. The observed and expected values were compared using a T-test.

2.3 Results

2.3.1 RNAi Screen Reveals Three Important Pathways for the Survival and Cisplatin Sensitivity of A549 Cells

Cisplatin-potentiating targets, shown in Figure 2.2, were identified using the “gene score”, which considered two selection criteria: 1) gene knockdown alone had to exhibit little or no effect on cell viability (survival index) and 2) gene knockdown sensitized the cells to the cytotoxic effects of CDDP. The survival index is a measure of the lethality of the gene knockdown alone while the sensitization or potentiation effect is a measure of the difference in cell viability in the presence and absence of low-dose (IC_{10}) CDDP. These two factors give rise to the Gene Score (see Methods Section 2.2.5) which was used to rank all potential targets.

The results summarized in Figure 2.3 identify the most lethal genes; genes that when silenced alone resulted in a low survival index, i.e. silencing resulted in at least 70% loss in cell viability in the absence of additional treatments. The top 100 CDDP enhancers and the 100 most lethal genes were analyzed separately using Pathway Studio. The resulting pathways were compared and three pathways: Hedgehog, Insulin Action, and Cell Cycle Regulation, were found to have the most impact on A549 cell viability and CDDP sensitivity (Table 2.1).
Figure 2.2. Top 100 hits from the genome-wide siRNA screen. A summary of the top hits identified from the siRNA screen is presented as a heat map ranked by the gene score which takes into account both the survival index and the level of CDDP activity enhancement. The survival index is a measure of cell viability relative to negative controls and the amount of cell death induced in the presence of both gene knockdown and CDDP is displayed in the second column. The difference between the first two columns is illustrated in the sensitization column.
Figure 2.3. Most lethal targets identified in the siRNA screen. The 100 genes with the lowest gene score are displayed here, ranked by lowest to highest survival index. While few of these genes may still sensitize A549 cells to CDDP treatment, gene knockdown in the absence of CDDP would cause at least 70% loss in cell viability.
Table 2.1. Pathways essential for A549 cell survival and cisplatin sensitivity

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Gene Name</th>
<th>Gene Knockdown (% Survival)</th>
<th>Gene Knockdown + Cisplatin&lt;sup&gt;a&lt;/sup&gt; (%Survival)</th>
<th>Gene Score Rank&lt;sup&gt;b&lt;/sup&gt;</th>
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</thead>
<tbody>
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<td>MBTD1</td>
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<td>UBE2M</td>
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<td>SUV39H1</td>
<td>27.69</td>
<td>30.21</td>
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</table>

<sup>a</sup>The cisplatin dose used caused no observable effects (<10% loss in cell viability) under non-silencing conditions.

<sup>b</sup>*Gene Score Rank* is the ranking of genes as cisplatin potentiators based on the Gene Score (calculation described in Methods). A lower value for the rank suggests that the target when silenced enhances the cytotoxic effects of cisplatin.
2.3.2 Analysis and Validation of Microarray Data

Unlike other microarray studies completed using high drug doses (e.g. IC\textsubscript{50}); the microarray study presented here was completed using a NOEL of CDDP (e.g. IC\textsubscript{10}). The NOEL dose was chosen to mimic a condition where cancer cells are exposed to a sub-lethal concentration of the drug with the assumption that these cells will develop adaptive responses that protect them in the short term and select for resistance in the long term. These responses should be detectable at the molecular level including changes in mRNA expression. Our microarray data suggest that 151 genes were differentially expressed following exposure to the NOEL of CDDP. Among the 151 genes, 50 were significantly down-regulated and 101 up-regulated (p<0.005) and have been summarized in Figure 2.4. The seven most up-regulated genes and the one most down-regulated gene were validated via quantitative RT-PCR (probes listed in Table A.1). All up-regulated genes were confirmed to be over-expressed following addition of the NOEL of CDDP (p<0.05) while the change detected in the selected down-regulated gene was not statistically significant (Fig. 2.4B-C). The log fold-change and the corresponding data from the siRNA screen for the up-regulated genes have been summarized in Table 2.2.
Figure 2.4. Differentially expressed genes in A549 following cisplatin treatment at its IC$_{10}$. The heat map represents the gene expression profile for the differentially expressed genes identified between untreated (blue) and CDDP-treated (orange) cells (A). Each column displays data from one microarray chip. Genes that were further validated by qPCR are highlighted with a black vertical line beside the gene. The fold-changes in mRNA expression are plotted in the box plot (B; blue = over-expression, red = under-expression after CDDP treatment) and the averaged values are tabulated from six individual experiments at each time-point (C). All changes observed were statistically significant (p<0.05) except for the gene FNTA.
Table 2.2 Over-expressed genes from Affymetrix Exon 1.0 Array matched with siRNA Screen Data

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Log Fold-Change</th>
<th>% Survival with CDDP</th>
<th>Survival Index</th>
<th>Potentiation</th>
<th>Gene Score</th>
<th>Whole Genome Rank</th>
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<tr>
<td>C2orf88</td>
<td>-0.3592</td>
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<td>65.64</td>
<td>6224</td>
<td>706</td>
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<td>92.93</td>
<td>66.97</td>
<td>6224</td>
<td>708</td>
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<sup>a</sup> Log-fold change = change in gene expression following CDDP treatment in the microarray study. All other data are collected from the siRNA screen for each corresponding gene.

<sup>b</sup> % Survival with CDDP = cell viability of target-silenced cells treated with low-dose (IC<sub>10</sub>) CDDP; presented as percentage relative to non-silencing and no CDDP controls.

<sup>c</sup> Survival Index = cell viability following gene knockdown, presented as % relative to untreated controls

<sup>d</sup> Potentiation is the difference in cell count between untreated and CDDP-treated when the gene is silenced; presented as % normalized to BRCA2 positive controls.

<sup>e</sup> Whole Genome Rank is the ranking of genes based on gene score.
2.3.3 *RRM2B* and *CABYR* Silencing Sensitized A549 and H460 Cells to Cisplatin Treatment

To identify CDDP activity enhancers, over-expressed genes from the microarray study that were also ranked within the top 10% of genes in the siRNA screen were identified. Five of these genes (ALDH3A1, RRM2B, CABYR, FHL2, and NINJ1) were selected for further validation based on evidence of their role in cancer (Table 2.3). The siRNA sequences used to suppress the expression of the selected genes for the validation studies are shown in Table 2.4. The level of gene knockdown was verified via RT-qPCR (Fig. 2.5A) and the validation studies were completed in two cell lines (A549 and H460). Based on the results summarized in Figure 2.5, ALDH3A1 knockdown was lethal in A549 cells (Fig. 2.5B), causing over 70% reduction in plating efficiency (PE) relative to the scramble control while in H460 cells, ALDH3A1 knockdown only reduced PE by 12%. In H460 cells, ALDH3A1 silencing in combination with CDDP resulted in a 46% reduction in PE relative to scrambled cells treated with CDDP (Fig. 2.5C). A smaller, albeit significant, decrease in PE was also observed in the ALDH3A1-silenced A549 cells treated with CDDP. On the other hand, FHL2 knockdown sensitized both A549 and H460 cells to a NOEL of CDDP, reducing the PE by 49.8% and 87.1%, respectively. However, the gene knockdown alone caused approximately 40% loss in viability in both cell lines. In a third scenario, although sensitization to CDDP was observed when NINJ1 was silenced in both cell lines, the effects were not statistically significant. Finally, RRM2B and CABYR knockdown in the cell lines appeared well-tolerated (< 25% reduction in clonogenicity; Fig. 2.5C) and in the presence of the NOEL of CDDP, the PE was further reduced by approximately 40% relative to the scramble control (Fig. 2.5C).
Table 2.3. Potential cisplatin activity enhancers selected from the siRNA screen and microarray gene expression studies and their known roles in cancer biology

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Relevance to Cancer</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRM2B</td>
<td>Ribonucleotide reductase M2 B</td>
<td>P53-inducible subunit of the human ribonucleotide reductase important for DNA repair; Role in NSCLC unknown but controversial in other cancer types:</td>
<td>[126, 127]</td>
</tr>
<tr>
<td>CAbYR</td>
<td>Calcium binding tyrosine phosphorylation regulated protein</td>
<td>Cancer-testis antigen; gene expressed in lung cancer tissues and patient sera; also shown to sensitize A549 and H460 cells to cisplatin and Taxol treatments in vitro and in vivo</td>
<td>[94, 128]</td>
</tr>
<tr>
<td>NINJ1</td>
<td>Nerve injury-induced protein 1</td>
<td>Adhesion molecule important for nerve regeneration; overexpressed in human cancer and induced by DNA damage in a p53-dependent manner</td>
<td>[129]</td>
</tr>
<tr>
<td>ALDH3A1</td>
<td>Aldehyde dehydrogenase 3 family, member A1</td>
<td>Aldehyde dehydrogenase involved in metabolism of xenobiotics; over-expressed in NSCLC; high expression confers resistance to nitrogen mustards</td>
<td>[130-133]</td>
</tr>
<tr>
<td>FHL2</td>
<td>Four and a half LIM domains 2</td>
<td>Interact with transcription factors and proteins involved in cancer development; de-regulated in various tumour tissues</td>
<td>[134]</td>
</tr>
</tbody>
</table>

Table 2.4. siRNA Sequences Used for Target Validation

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Stealth siRNA Sequences (5’ to 3’)</th>
<th>Sequence ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRM2B</td>
<td>GCA GUU AUG GCA GAA ACC ACA GUA A</td>
<td>HSS121295</td>
</tr>
<tr>
<td></td>
<td>GCC UGA UGU UCC AAU ACU UAG UAA A</td>
<td>HSS121296</td>
</tr>
<tr>
<td></td>
<td>CAU UGA GUU UGU AGC UGA CAG AUU A</td>
<td>HSS181703</td>
</tr>
<tr>
<td>CAbYR</td>
<td>CAG AAG GAA CGA CAC CAC AGA AGA A</td>
<td>HSS146764</td>
</tr>
<tr>
<td></td>
<td>ACA GAC ACA GAC GAG GAC AAU GUA A</td>
<td>HSS146765</td>
</tr>
<tr>
<td></td>
<td>GGU GAC AAA UGU GCU CCC UUU GGA A</td>
<td>HSS178124</td>
</tr>
<tr>
<td>NINJ1</td>
<td>ACG GGC CCA UCA ACG UGA ACC AUU A</td>
<td>HSS107188</td>
</tr>
<tr>
<td></td>
<td>GCC UGG UGU UCA UCG UGG UAG U</td>
<td>HSS107190</td>
</tr>
<tr>
<td></td>
<td>GGG UGC UGC UCA UCC UUG UCA A</td>
<td>HSS181529</td>
</tr>
<tr>
<td>ALDH3A1</td>
<td>GCC AAC GAU GUC AUC GUC CAC AUC A</td>
<td>HSS100373</td>
</tr>
<tr>
<td></td>
<td>AGG AGA GGU UCG ACC AUA UCC UGU A</td>
<td>HSS100374</td>
</tr>
<tr>
<td></td>
<td>CAG AAC CAA AUU GUG GAG AAG CUC A</td>
<td>HSS176687</td>
</tr>
<tr>
<td>FHL2</td>
<td>GCC UGA ACU GCU UGU GUG UGU A</td>
<td>HSS142018</td>
</tr>
<tr>
<td></td>
<td>CCU GCU AUG AGA AAC AAG AUG CCA U</td>
<td>HSS142019</td>
</tr>
<tr>
<td></td>
<td>CCC UGG CAC AAG GAG UGC UUC GUG U</td>
<td>HSS142020</td>
</tr>
</tbody>
</table>
Figure 2.5. Silencing RRM2B or CABYR sensitizes A549 and H460 cells to cisplatin (CDDP) treatment. Knockdown of each candidate target was confirmed via qPCR (A; *p<0.05 relative to scramble control). The representative image for each tested condition is displayed in B. The plating efficiency of each condition is plotted in separate graphs for each cell line (E; data shown as mean ± SEM; n=3; *p<0.05).

2.3.4 The INO80 Chromatin Remodeling and the Double Strand DNA Homologous Repair Pathways are Induced by Low-Dose Cisplatin and May Be Targeted to Improve Response to Platinum Treatments

To systematically compare the siRNA screen and the Affymetrix study at the pathway level, the siRNA screen hits were filtered by potentiation effect and survival index (see Methods 2.2.5). This resulted in a list of 480 genes that enriched for pathways involved with chromatin remodeling and DNA modification (Table 2.5). On the other hand, pathways enriched in the microarray study were primarily involved in apoptosis induction and various types of DNA repair (Table 2.6). A comparison of significant pathways (p-value < 0.05) from the siRNA screen and microarray study revealed enrichment in the INO80 chromatin remodeling pathway and the double strand DNA homologous repair pathway. Figure 2.6 illustrates the two pathways enriched in both the siRNA screen and the
microarray, where hits identified from the siRNA screen are highlighted in purple and differentially expressed genes are shown in green.

To validate some of the CDDP-potentiating pathways uncovered by our studies, we turned to the simple model organism *Saccharomyces cerevisiae* (yeast). As expected, yeasts deficient in homologous recombination (*rad52Δ*) or nucleotide excision repair (*rad1Δ*) were highly sensitive to CDDP (Figure 2.7). In addition, *RAD18*, *CHTF8* and *H2AV* genes predicted by our siRNA screen were also recapitulated as CDDP hypersensitive when the corresponding yeast gene deletion was tested. Interestingly, deletion of yeast INO80 accessory subunits *IES1-6* did not cause CDDP sensitivity in yeast.

**Table 2.5. Pathway enrichment analysis of cisplatin-potentiating gene targets from the siRNA screen**

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Associated genes</th>
<th>p-value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>INO80 Chromatin Remodeling</td>
<td>INO80D, DCLRE1C, SMARCD3, BAZ1A, KAT2A, KAT6A, HDAC9, CDT1, PMS2, ORC1, RAD18, CHTF8, H2AFV, NAP1L2, RFC3, MBTD1</td>
<td>0.0015169</td>
</tr>
<tr>
<td>Histone and DNA Methylation</td>
<td>SMARCD3, BAZ1A, KAT2A, KAT6A, HDAC9, SALL1, CDT1, ORC1, CHTF8, H2AFV, NAP1L2, RFC3, MBTD1</td>
<td>0.0032743</td>
</tr>
<tr>
<td>Histone Acetylation</td>
<td>SMARCD3, KPNA2, BAZ1A, KAT2A, IPO5, SORBS2, KAT6A, HDAC9, SALL1, H2AFV, NAP1L2, MBTD1</td>
<td>0.0049853</td>
</tr>
<tr>
<td>Hedgehog Pathway</td>
<td>IHH, DCLRE1C, SMARCD3, CUL4A, POT1, ANAPC1, BAZ1A, KAT2A, KAT6A, HDAC9, CDT1, PMS2, ORC1, RAD18, CHTF8, H2AFV, NAP1L2, WNT16, RFC3, KDM2A, MBTD1</td>
<td>0.0092622</td>
</tr>
<tr>
<td>TRRAP/TIP60 Chromatin Remodeling</td>
<td>Gas41, DCLRE1C, SMARCD3, BAZ1A, KAT2A, KAT6A, HDAC9, PMS2, RAD18, H2AFV, NAP1L2, HELQ, MBTD1</td>
<td>0.01193</td>
</tr>
<tr>
<td>Histone Ubiquitination</td>
<td>SMARCD3, BAZ1A, KAT2A, KAT6A, NAE1, HDAC9, CTR9, H2AFV, NAP1L2, UBE2G1, MBTD1</td>
<td>0.0131073</td>
</tr>
<tr>
<td>Sister Chromatid Cohesion</td>
<td>ANAPC1, CDT1, DLGAP5, ORC1, CHTF8, RFC3, TACC2, C8G, CR2, CFP</td>
<td>0.0160821</td>
</tr>
<tr>
<td>Alternative Complement Pathway</td>
<td></td>
<td>0.0236879</td>
</tr>
<tr>
<td>Double Strand DNA Homologous Repair</td>
<td>DCLRE1C, CDT1, PMS2, ORC1, RAD18, CHTF8, RFC3</td>
<td>0.0252954</td>
</tr>
<tr>
<td>AGER -&gt; CREB/SP1 signaling</td>
<td>RPS6KA3, PIK3R1, S100A1</td>
<td>0.027402</td>
</tr>
<tr>
<td>Co-translational ER Protein Import</td>
<td>RPS6KA3, P4HB, HSPH1, SEC63, RPS6KA6, RPS6KC1, RPS28, NACA2, TMX3, MRPLS1</td>
<td>0.0327461</td>
</tr>
<tr>
<td>SRCAP Chromatin Remodeling</td>
<td>Gas41, SMARCD3, BAZ1A, KAT2A, KAT6A, HDAC9, H2AFV, NAP1L2, MBTD1</td>
<td>0.0351599</td>
</tr>
<tr>
<td>Histone Sumoylation</td>
<td>SMARCD3, BAZ1A, KAT2A, KAT6A, HDAC9, H2AFV, NAP1L2, MBTD1</td>
<td>0.0446793</td>
</tr>
<tr>
<td>Metabolism of triacylglycerols</td>
<td>PNLP1RP3, AGPAT9, DAK</td>
<td>0.0474868</td>
</tr>
</tbody>
</table>

<sup>a</sup>The p-value is derived from the Pathway Studio software that determines the likelihood of the pathway being enriched from a random gene list of the same size.
<table>
<thead>
<tr>
<th>Pathway</th>
<th>Associated genes</th>
<th>p-value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis</td>
<td>MDM2, FAS, BAX, TNFRSF10B, TNFRSF10D</td>
<td>0.000478709</td>
</tr>
<tr>
<td>Apoptosis Regulation</td>
<td>XPC, MDM2, FAS, TNFRSF10B, TNFRSF10D, TNFSF9, RRM2B, DDB2, EDA2R</td>
<td>0.00220469</td>
</tr>
<tr>
<td>Double Strand DNA Homologous Repair</td>
<td>XPC, RRM2B, POLH, DDB2</td>
<td>0.00406061</td>
</tr>
<tr>
<td>Single-Strand Base Excision DNA</td>
<td>XPC, RRM2B, DDB2</td>
<td>0.00946967</td>
</tr>
<tr>
<td>Repair</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single-Strand Mismatch DNA Repair</td>
<td>XPC, RRM2B, DDB2</td>
<td>0.0103586</td>
</tr>
<tr>
<td>Direct DNA Repair</td>
<td>XPC, RRM2B, DDB2</td>
<td>0.0115367</td>
</tr>
<tr>
<td>Histone Phosphorylation</td>
<td>XPC, RRM2B, DDB2, PAK3</td>
<td>0.0120578</td>
</tr>
<tr>
<td>Double Strand DNA Non-Homologous</td>
<td>XPC, RRM2B, DDB2</td>
<td>0.0133127</td>
</tr>
<tr>
<td>Repair</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFR -&gt; NF-kB signaling</td>
<td>FAS, TNFRSF10B</td>
<td>0.0151032</td>
</tr>
<tr>
<td>TNFR -&gt; AP-1/ATF/TP53 signaling</td>
<td>FAS, TNFRSF10B</td>
<td>0.0151032</td>
</tr>
<tr>
<td>TNFR -&gt; CREB/ELK-SRF signaling</td>
<td>FAS, TNFRSF10B</td>
<td>0.0189529</td>
</tr>
<tr>
<td>Cell Cycle Regulation</td>
<td>XPC, MDM2, FAS, CDKN1A, KITLG, TNFRSF10B, TNFRSF10D, TP53INP1, TNFSF9, RRM2B, TNS4, POLH, DDB2, DUSP4, PAK3, EDA2R, MBD3L1</td>
<td>0.0190788</td>
</tr>
<tr>
<td>INO80 Chromatin Remodeling</td>
<td>XPC, RRM2B, POLH, DDB2, MBD3L1</td>
<td>0.0211387</td>
</tr>
<tr>
<td>KIT -&gt; STAT signaling</td>
<td>KITLG</td>
<td>0.029861</td>
</tr>
<tr>
<td>Sialophorin -&gt; CTNNB/MYC/TP53</td>
<td>MDM2</td>
<td>0.029861</td>
</tr>
<tr>
<td>signaling</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfur metabolism</td>
<td>SULT1C3, SULT1A2</td>
<td>0.0332819</td>
</tr>
<tr>
<td>TNFRSF6 -&gt; FOXO3A signaling</td>
<td>FAS</td>
<td>0.0347648</td>
</tr>
<tr>
<td>Metabolism of glucocorticoids and</td>
<td>SULT1C3, AKR1C1</td>
<td>0.0379581</td>
</tr>
<tr>
<td>mineralcorticoids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adipocytokine Signaling</td>
<td>FAS, TNFRSF10B, UGT2B17, TNFRSF10D, TNFSF9, DUSP4, ALDH3A1, EDA2R</td>
<td>0.0401247</td>
</tr>
<tr>
<td>EctodysplasinR -&gt; LEF1 signaling</td>
<td>EDA2R</td>
<td>0.0493525</td>
</tr>
</tbody>
</table>

<sup>a</sup>The p-value is derived from the Pathway Studio software that determines the likelihood of the pathway being enriched from a random gene list of the same size.
Figure 2.6. Pathways enriched in both the siRNA screen top hits and the microarray differentially expressed genes. Of the siRNA screen hits and the microarray gene list, the double stranded homologous repair (A) and the INO80 chromatin remodelling (B) pathways were enriched in both datasets. The schematic diagram of the two pathways are displayed on the left while the entities from the siRNA screen (purple) and the microarray study (green) that map to these pathways are illustrated based on cellular localization (right). The pathway illustrations were generated using the Pathway Studio software following pathway enrichment analyses.
Fig 2.7. **Fitness measurements for cisplatin treated yeast mutant strains.** WT fitness (white bar) was used to predict the effects of CDDP on mutant strains assuming a simple multiplication of the mutant fitness defect and the effect of CDDP on WT. Significantly lower fitness values were observed for strains deleted for RAD1, RAD18, RAD52, CTF8, MRE11 or HTZ1 (Holm-Bonferroni corrected p-value <0.05) but not for other mutants tested. Raw p-values are indicated above each comparison. Human gene orthologues of each yeast mutant are indicated below. Errors bars are standard error of the mean.

### 2.4 Discussion

The siRNA screen was designed to identify genes that when inhibited would sensitize NSCLC cells to CDDP treatment. Prior to the whole genome screen (WGS), a preliminary kinome screen was conducted to establish the screening parameters, with some of the targets identified being further validated (see Chapter 3). Several other groups have previously conducted siRNA screens for CDDP modulators. Similar to the study presented here, Bartz *et al.* performed a genome-wide siRNA screen; however, the study evaluated effects in HeLa cells. All other published studies appear to focus on the kinases (778 genes) or the druggable genome (7000 genes) [92, 98, 114, 135]. While most published screens utilized metabolic assays to determine cell viability, the use of nuclei count in this study
avoids inaccuracies caused by alterations in cellular metabolism due to a particular gene knockdown [97]. The high content screen used in our studies also allowed us to establish whether CDDP treatment caused morphological changes in the cells not normally considered in metabolic assays.

This study is the first to explore the responses of NSCLC cells exposed to a NOEL of CDDP. Nevertheless, there is still considerable overlap between our screen and those performed by other groups despite differences in cell lines, siRNA library, CDDP dosage, time point, and endpoint assay used [135]. For instance, BRCA1, BRCA2, RAD18, REV1L, and RFWD3 were identified in the study reported by Bartz et al. and these genes were also found to potentiate CDDP activity in our screen. CALM1, NRGN and PTK9L were identified in the study reported by Arora et al. and STK16 was identified in the study reported by Swanton et al. These genes also significantly enhanced CDDP activity in our study. Interestingly, CHEK1 was shown to enhance CDDP sensitivity in the screens reported by Bartz and Arora’s groups; however, our results were consistent with those reported by Nijwening et al., which suggested that CHEK1 inhibition alone was lethal. We believe that our experimental design and the use of a gene score to select gene candidates have allowed us to generate a distinct list of gene candidates that may be targeted to enhance CDDP sensitivity.

When comparing pathways derived from the strongest CDDP potentiators and the most lethal gene knockdowns, the hedgehog, insulin action, and cell cycle regulation pathways were found to be enriched in both lists, suggesting that these pathways play significant roles in mediating cell survival and response to CDDP treatments. The insulin action and cell cycle regulation pathways are known to be associated with cancer progression and resistance [136, 137]. Activation of the insulin signaling pathway results in downstream activation of oncogenic pathways such as the phosphatidylinositol 3-kinase (PI3K) and the MAPK pathways which are important for cell growth and proliferation [138]. These pathways are also suggested to be important targets for overcoming chemoresistance [139, 140]. The hedgehog pathway (Hh) is activated in multiple types of cancers including NSCLC [141-143]. This pathway is associated with cancer growth, metastasis, and drug resistance and is involved in crosstalk with other commonly deregulated pathways in cancer such as the Notch and Wnt signaling pathways [141, 144]. Currently, the Hh pathway inhibitor
vismodegib is approved for use in basal cell carcinoma while several other inhibitors are still being tested in clinical trials. Vismodegib and BMS-833923 are both being evaluated in combination with a platinum-containing doublet for treatment in patients with SCLC. In NSCLC, the Hh pathway is thought to be involved in epithelial-to-mesenchymal transition [145]. Furthermore, Tian et al. have demonstrated that the combination of vismodegib and CDDP is more cytotoxic in NSCLC cells than either treatment alone; a result which suggests synergistic interactions [146].

It was anticipated that results from the siRNA screen would complement results from the gene expression studies, leading to the identification of pathways/targets that are up-regulated when cells are exposed to the NOEL of CDDP and, when silenced, cause the NOEL to become lethal. Here, we validated five upregulated genes that were identified as “hits” in the siRNA screen using a clonogenic assay: an assay that assessed the long-term effects of the gene knockdown in the presence and absence of an empirically derived NOEL of CDDP. It is important to note that the clonogenic assay assesses tumor cell survival over 14 days in contrast to the high content screen which evaluated effects after 3 days. Additionally, the siRNA sequences used in our validation differed from those used in the siRNA screen to ensure that the effects observed were not sequence-specific. A549 and H460 cells were used for our validation studies as they are chemotherapy-naïve and the primary objective is to improve CDDP treatment in the first line setting. Note that both cell lines are KRAS mutants that harbour wild-type p53. The validation results summarized in Figure 2.5 illustrated four main points. First, the CDDP-potentiating effects associated with gene silencing could be subtype or cell context-specific. For instance, ALDH3A1 knockdown caused lethality in A549 cells but not in H460 cells. Since ALDH3A1 is the only target for which a small molecule inhibitor (CB29) is commercially available, we attempted to validate the target by adding the maximal non-toxic dose of CB29 in combination with different concentrations of CDDP. However, due to solubility issues and the need of using DMSO, which is known to inactivate CDDP and other platinum-based therapeutics, the validation studies could not be completed in a meaningful way [147]. Second, while most screening approaches rely on short-term (3-day) assays, target validation should be conducted using long-term assays such as the clonogenic assay as a means of eliminating hits such as FHL2 where gene knockdown appeared to be non-essential over a short-term
assay but was found to be lethal when effects on long-term cell viability were evaluated. Third, lead targets should be further evaluated in a range of cell lines to ensure that hits such as NINJ1 are sensitizing NSCLC cells to CDDP to a sufficiently substantial extent that further validation in vivo is worth pursuing. Fourth, RRM2B and CABYR suppression enhanced CDDP activity in both A549 and H460 cells. A study conducted on 39 cervical cancer tumour samples revealed that RRM2B is consistently up-regulated in response to chemo-radiation where CDDP was used as a radiosensitizer, suggesting that RRM2B may be a clinically relevant target for chemosensitization [148]. On the other hand, it has recently been shown that CABYR suppression enhances the cytotoxic effects of CDDP in A549 and H460 [94]; consistent with our findings. Interestingly, CABYR is not normally expressed in the lung but has been detected in lung cancer tissues from patients [94, 128]. It is therefore a clinically relevant and promising target. To determine whether CABYR knockdown only sensitized lung cancer cells to low-dose CDDP, we performed additional clonogenic assays using a range of CDDP concentrations (Figure A.1). Our results suggest that suppressing CABYR expression would enhance CDDP activity significantly at low as well as high doses of CDDP. To determine whether RRM2B and CABYR should be further pursued as therapeutic targets, future validation work could expand to additional cell lines with different genetic background or driver mutations as well as efficacy assessments in vivo.

With regards to pathway analyses, most of the differentially expressed genes we identified were up-regulated [149, 150] and were mainly important for apoptosis and DNA repair, consistent with other microarray studies on CDDP response. These results were further confirmed via GO analysis of the up-regulated genes (Table A.2) and are not unexpected since CDDP is a DNA damaging agent. DNA repair must be effective and efficient for the cells to survive exposure to the agent, even when the dose is low. Importantly, hierarchical clustering revealed several groups of up-regulated genes enriched in p53 signalling (p = 2 x 10^{-6}), six of which are amongst the seven most over-expressed genes (Figure A.2). This finding is consistent with another study that demonstrated activation of the p53 signaling pathway in ovarian cancer in response to CDDP treatment [150].

In our pathway analyses, the INO80 chromatin remodeling pathway and the DNA double strand homologous repair (HR) pathway were enriched in both datasets. When attempting
to verify these findings in yeasts, mutants with deletions in some of the components of these pathways were found to be hypersensitive to CDDP; however, deletion of one of the INO80 accessory subunit did not sensitize yeast to CDDP treatment. Nonetheless, the yeast and human INO80 complexes have different subunit compositions and the INO80D gene, which was identified in our siRNA screen (Table 2.5), is not conserved in yeast. It is possible that the catalytic activity of INO80A is required to modulate CDDP treatment response or that INO80D has a specific role in the response of human cells to CDDP. Here, we have elucidated some components of the double-stranded homologous DNA repair and the INO80 chromatin remodeling pathways in a simplified eukaryotic system. To further understand the importance of the INO80 chromatin remodeling complex in modulating CDDP treatment response, future studies will require testing each human subunit and performing functional assays to identify the subunit(s) that could be targeted to induce selective killing of cancer cells in the presence of low-dose CDDP.

The importance of DNA repair in CDDP treatment response is well-documented [151-154]. While ATP-dependent chromatin remodeling is known to be important for gene transcription, previous studies have also demonstrated that the INO80 chromatin remodeling complex (Fig. 2.6B) interacts with γ-H2AX in the presence of DNA damage and is directly involved in the repair of double-stranded DNA breaks [155, 156]. A functional HR repair pathway plays a role in repairing CDDP-induced DNA damage [157, 158]; however, when defective, the pathway has an increased sensitivity to DNA damaging agents [159]. It is only in recent years that chromatin remodeling complexes gained recognition as being important for repairing double-stranded breaks. Our findings further suggest that the INO80 chromatin remodeling pathway is induced in response to CDDP and may be targeted to enhance tumor cells’ sensitivity to CDDP [160].

As mentioned above, many of the differentially expressed genes identified are involved in DNA repair. Although the nucleotide excision repair (NER) pathway is known to be a major DNA repair pathway involved in removing CDDP-DNA adducts, it was not identified as a hit in the current study primarily because of the lack of overexpression in NER genes in response to low-dose CDDP [161-164]. A previous study in melanoma has also demonstrated that majority of NER genes are not upregulated at 6 and 24 hours following CDDP treatment but CDDP resistance was still observed as the cancer cells had a higher
basal level of NER gene expression than normal cells [165]. The importance of NER in CDDP treatment response is still evident in our siRNA screen, however, as about 1/3 of the NER genes in our screen were found to sensitize A549 cells to CDDP when silenced via siRNA (Table A.3), consistent with other studies in the literature [135, 161, 166, 167]. Therefore, while we have identified two lead pathways based on both the siRNA screen and the microarray study, pathways identified from either study alone could also be further explored as CDDP-sensitizers.

The primary objective of the siRNA screen and the microarray study was to identify genes that are up-regulated in response to a NOEL of CDDP, which if inhibited, would enhance the cytotoxic effects of the drug. Aside from genetic heterogeneity within the tumour which results in different sensitivities of tumour sub-populations to drug treatment, we are aware that tumour cells that are exposed to sub-lethal doses of chemotherapeutics are also likely to be distant from blood vessels, having limited access to nutrients and oxygen because of the poorly organized tumour vasculature [112, 168]. We believe that future studies could involve conducting a similar siRNA screen under hypoxic conditions and comparing the list of hits with the data generated from the current study. It will also be of interest to examine whether the sensitization to CDDP treatment observed here would be weakened, maintained, or enhanced under hypoxic and/or starvation conditions to determine whether the gene targets or pathways identified here should be further pursued from a pharmaceutical development perspective. Furthermore, potent inhibitors will be needed to successfully target the gene products as the new inhibitors may also encounter similar limitations to CDDP in penetrating certain regions of the tumour. Drug delivery technologies may also be useful in increasing the availability of these agents for better drug distribution as well as in decreasing the rate of clearance [112, 169, 170]. Overall, the studies presented here highlight two important points: First, cancer cells react to cytotoxic drugs even when exposed to a dose that triggers no observable effect over 72hrs. This reaction involves multiple changes in gene expressions and activation of pathways such as those involved in chromatin modification and DNA repair. These results could help guide the development of targeted therapeutics to be used in combination with CDDP to suppress survival responses induced within cancer cells when first exposed to sub-lethal concentrations of the drug. Second, pathways involved in INO80 chromatin remodeling and
repairing of double strand breaks are up-regulated in response to low-dose CDDP and should be targeted in chemo-naive cells to improve the effectiveness of CDDP treatment.
3. 3’-Phosphoadenosine 5’-Phosphosulfate Synthase 1 (PAPSS1) Knockdown Sensitizes Non-Small Cell Lung Cancer Cells to DNA Damaging Agents

3.1 Introduction

With a 5-year survival rate of 16%, lung cancer continues to be the leading cause of cancer-related deaths [171, 172]. Although lung cancer is primarily caused by smoking, approximately 25% of worldwide sufferers never smoked (lifetime exposure of <100 cigarettes) and there appears to be a rise in the incidence of non-smoking related lung cancers worldwide [173]. Nearly 85% of all lung cancers are attributed to non-small cell lung cancer (NSCLC), of which 65-80% patients are diagnosed with an inoperable, locally advanced or metastatic disease [1, 2]. Platinum-based combination chemotherapy, consisting of carboplatin or cisplatin (CDDP) in combination with a second drug (gemcitabine, paclitaxel, or vinorelbine), has been the standard treatment for advanced NSCLC patients for the past two decades [1, 174]. In recent years, movement towards personalized medicine resulted in the development and use of EGFR, ALK, and other tyrosine kinase-targeting inhibitors (TKIs) as first-line treatments for patients whose tumors harbor these known driver mutations. This treatment strategy is associated with more favorable toxicity profiles and improved progression-free survival over standard chemotherapy alone but it has been difficult to demonstrate an overall survival advantage, and resistance to TKIs have been noted [175, 176]. Furthermore, only a small population of NSCLC patients in western countries has these mutations and hence, most patients still rely on platinum-based treatments in the first line setting [177].

Based on the clinical data from various NSCLC-focused clinical trials it is certain that CDDP is unlikely to be replaced in the first line setting; at least for the majority of patients [178]. We argue here that one of many reasons for the lack of improvement in treating NSCLC concerns the fact that new drugs are not developed in the context of CDDP use in a first line setting. For this reason, our laboratory embarked on a siRNA screen in combination with low-dose CDDP (IC10) in an attempt to identify targets that potentiate the therapeutic effects of CDDP when the cells are first exposed to the drug. One of the premises behind the
design of this screen was the belief that chemotherapy-naïve lung cancer cells that are not exposed to lethal CDDP concentrations \textit{in vivo} will develop cytoprotective responses. If such cytoprotective responses occur, then it will be possible to develop strategies designed to inhibit these responses. This, in turn, will be expected to increase the potency of CDDP when first used to treat chemo-naïve NSCLC patients. A second premise concerns the potential for the screen to identify synthetic-sick interactions where an ineffective dose of CDDP could prove very effective when added to a cell population where selected genes have been silenced. In the previous chapter, the siRNA screen was described and hits were selected based on differential gene expression following treatment with low-dose CDDP. This chapter reports on validation studies completed on a top hit identified from the siRNA screen alone. Our results demonstrate, for the first time, that silencing of 3’-phosphoadenosine 5’-phosphosulfate (PAPS) synthase 1 (PAPSS1), a bi-functional enzyme that synthesizes the universal sulfate donor PAPS \cite{179}, can enhance CDDP activity in NSCLC cell lines by inducing apoptosis and G1/S phase cell cycle arrest. Importantly, PAPSS1 silencing also enhances the activity of radiation, other platinum agents, topoisomerase I inhibitors, but not topoisomerase II inhibitors or microtubule-targeted drugs.

3.2 Methods

3.2.1 Cell Culture and Reagents

The NSCLC cell lines A549, H460, H1703, and H358 were obtained directly from Dr. John Minna’s laboratory (Dallas, TX) and maintained at 37°C and 5% CO\textsubscript{2} in RPMI 1640 (Gibco) supplemented with 2 mM L-glutamine (Gibco) and 10% fetal bovine serum (Gibco). Human Bronchial Epithelial Cells (Cell Applications) were maintained in Bronchial/Tracheal Epithelial Growth Medium (Cell Applications) at 37°C and 5% CO\textsubscript{2}. HBEpC cells beyond passage 3 were not used for experiments. PAPSS1 (1:1000) primary antibody was obtained from Abcam while β-Actin (1:50000), cleaved caspase-3 (1:1000), Cyclin A2 (1:2000), Cyclin E1 (1:1000), and cleaved PARP (1:1000) primary antibodies were purchased from Cell Signaling Technology. The chemotherapeutics CDDP, carboplatin, irinotecan, topotecan, and docetaxel were obtained as ready-to-inject solutions from Hospira.
Epirubicin and doxorubicin were obtained from Pfizer while oxaliplatin and mitomycin C were purchased from Sanofi Aventis and Novopharm respectively.

### 3.2.2 siRNA Kinome and Genome Screens

The preliminary kinase screen (PKS) and genome-wide siRNA screen were performed as described in the previous chapter (Methods 2.2.4 and 2.2.5). The screen results were validated through three independent experiments where the top 20 kinases from the PKS were targeted with a pool of three different Stealth siRNA duplexes (Life Technologies) using the same methodologies as the screens. Additional validation work was performed by generating CDDP dose response curves with the top five targets: cells were stained with Hoechst for total nuclei count and ethidium homodimer I (Biotium) for dead cells at 72 hours following CDDP treatment and imaged with the IN Cell Analyzer after a 20-minute incubation at 37°C. Cells were classified as “dead” if they showed >30% overlap of the two stains. Cell counts were performed using the IN Cell Developer Toolbox 1.9 software as described earlier.

### 3.2.3 siRNA Transfection for PAPSS1 Validation Studies Using Multiple NSCLC Cell Lines

The indicated NSCLC cells were seeded manually in 384-well plates at 50 µL/well of OPTI-MEM reduced serum media (Life Technologies) supplemented with 4% FBS and 2 mM L-glutamine. Briefly, RNAiMAX was diluted in OPTI-MEM and complexed with a pool of three different Stealth siRNA sequences targeting PAPSS1 (HSS113394, HSS189820, HSS189821; Life Technologies) for 20 minutes and then added to the cells. The Stealth RNAi negative control kit (Life Technologies) was used as scramble control.

### 3.2.4 RNA Extraction and RT-qPCR

Cells were seeded in 6-well plates and transfected with the indicated siRNAs as described above. Total RNA was extracted 48 hours post-transfection using the RNeasy Mini Kit (Qiagen) and quantified using the ND-1000 Spectrophotometer (NanoDrop Technologies). The QuantiTect Reverse Transcription Kit (Qiagen) was used to eliminate genomic DNA and to synthesize cDNA from 1 µg of total RNA. Quantitative Real-time PCR was performed
in triplicates via the 7900HT system (Applied Biosystems) using 2X TaqMan fast advanced master mix and 20X Taqman PAPSS1 (Hs00968937_m1) and GAPDH (hCG2005673) probes (Applied Biosystems) as per manufacturer's instructions. Data were analyzed using the SDS 2.2 software (Applied Biosystems) and the relative messenger RNA quantity was calculated using the ddCt method with GAPDH as the endogenous control.

3.2.5 SDS-PAGE and Western Blot Analysis

All buffer chemicals were obtained from Sigma-Aldrich. Cells were cultured in 6-well plates and transfected with siRNA the following day. At 72 hours post-transfection, cells were lysed with buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.25% sodium deoxycholate, 1% NP-40, 0.1% SDS, 1 mM EDTA and Mini Protease Inhibitor Cocktail tablets (Roche Diagnostics). Cellular lysates were clarified (20 min at 14000g) and protein concentrations were determined using the BCA Protein Assay Kit (Pierce). Lysates (30 µg) were separated by SDS-PAGE on a 4-12% Bis-Tris gel (Life Technologies) and transferred to 0.2 µm nitrocellulose membranes using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories). The membranes were blocked for 1 hour (5% skim milk in TBST [Tris buffered saline + Tween: 20 mM Tris-base, 140 mM NaCl, 0.1% Tween 20]) and incubated in blocking solution with primary antibodies (1:1000) overnight at 4°C. The membranes were washed with TBST (3 x 5 min) and then incubated with horseradish peroxidase-conjugated secondary antibodies (Promega) at room temperature for 1 hour (1:10000 for β-actin and 1:5000 for all others). The blots were then washed with TBST as before, developed using the Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific), and visualized using the ChemiDoc MP System and the ImageLab imaging software (Bio-Rad). Protein quantification was performed using the ImageLab software.

3.2.6 Flow Cytometry

Cells were plated and transfected in 6-well plates at 0.094 fmol siRNA per cell. At 24 hours post-transfection, cells were treated with the indicated doses of CDDP. At the indicated time points, the supernatant and adherent cells harvested by trypsinization were collected. For cell cycle analysis, the cells were washed with HBSS (Hank’s Balanced Salt Solution; Stem Cell Technologies) and fixed in 70% ethanol at 10^6 cells/mL for 1 hour on ice followed by an overnight incubation at -20°C. Cell pellets were collected by centrifugation
and then stained in PBS buffer containing 50 µg/mL propidium iodide (Invitrogen), 1 mg/mL RNase A (Sigma), and 0.1% Triton X-100 (Bio-Rad) for 15 min at 37°C followed by 1 hour on ice. To assess DNA damage, transfected cells were treated with CDDP or topotecan for 48 hours, and then fixed and stained with anti-γH2AX antibody and counterstained with Sytox® green nucleic acid stain according to manufacturer’s instructions included in the Apoptosis, DNA damage, and Cell Proliferation Kit (BD Pharmingen). A 24-hour treatment with 250 nM of topotecan was used as a positive control. Data were acquired and analyzed using the FACSCalibur flow cytometer and the WinMDI 2.9 software respectively. Based on Sytox® green fluorescent intensity, cells with less than 2N DNA were excluded from analysis of γH2AX expression.

### 3.2.7 Immunofluorescence Staining and High Content Analysis

Cells were seeded in a 96-well plate and transfected as described above. Cells were treated with different concentrations of CDDP or topotecan for 48 hours, after which the cells were fixed and permeabilized using the BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD Pharmingen) as per manufacturer’s instructions. Non-specific binding was blocked by incubating fixed cells with 100 µL of BD Pharmingen™ Stain Buffer per well for 30 minutes at room temperature. H2AX phosphorylation was labeled using the Alexa Fluor 647 Mouse anti-γH2AX (Ser139) antibody (BD Pharmingen) as per manufacturer’s instructions and the nuclei were counter-stained with 2 µg/mL of Hoechst 33342. Nine imaging fields/well were acquired using the IN Cell Analyzer 2200 (GE Healthcare) equipped with 20x objective and the data were analyzed using the IN Cell Analyzer Workstation 3.7 software (GE Healthcare).

### 3.2.8 Colony Formation Assay

Colonies formation assays were performed as described in Chapter 2 (Methods 2.2.9). Briefly, A549 cells were transfected in 6-well plates. After 24 hours, the cells were exposed to an empirically determined IC_{10} (0.71 µM) of CDDP for 24 hours, harvested by trypsinization, and then seeded at 500 cells per well in triplicate. The cells were then incubated at 37°C, 5% CO_{2} for 14 days without disturbance. Colonies were fixed with 6.25% glutaraldehyde (Sigma) and stained with 0.5% crystal violet (Sigma) for 30 minutes, washed with distilled water, dried overnight, and counted the next day. Plating efficiency
(PE) was defined as the percentage of trypan blue excluding cells that formed colonies of >50 cells (PE = [(no. of colonies formed/no. of cells seeded) x100%]). For radiation studies, cells were transfected for 48 hours and then irradiated at 10^6 cells/mL at the indicated doses (Pantak Seifert X-ray Systems, Test Equipment Distributors). Cells were then plated in triplicate to achieve 100-500 colonies 12 days later. Cells were stained with aqueous malachite green and dried overnight prior to colony counting. The surviving fraction (SF), plotted against the radiation dose, was calculated using the equation SF = [no. of colonies formed after treatment/ (no. of cells seeded x PE)].

3.2.9 Dose Response Curves

With the exception of the radiation study (see colony-formation assay methods), all dose response curves were conducted in 384-well plates. Cells were exposed to various concentrations of selected agents 24 hours following transfection. Cell viability was determined 72 hours post-treatment using the IN Cell Analyzer as described above.

3.2.10 Statistical Analysis

Dose response curves were plotted using Prism 6.0 (GraphPad Software) as mean ± SEM from at least three independent experiments. IC_{50} values were interpolated from the fitted curves and compared for statistical differences using one-way ANOVA followed by Tukey adjustments (top five targets) or the Student’s t-test (scramble vs. PAPSS1). Student’s t-test was used to statistically compare the differential PAPSS1/GAPDH mRNA expressions and the differences in IC_{50} values of various chemotherapeutics between Scramble and PAPSS1 siRNA-transfected cells. Radiation data were fitted in the linear-quadratic model in the SPSS 22 statistical software. P-values <0.05 was considered statistically significant.

3.3 Results

3.3.1 siRNA Screens Identified PAPSS1 as a Target Improving Cisplatin Activity when Silenced

A Preliminary Kinome Screen (PKS) comprising 640 kinases was performed prior to the Whole Genome Screen (WGS) to establish all screening parameters. Cisplatin-potentiating
candidates were identified using two selection criteria: 1) gene knockdown must have little or no impact on viable cell count in the absence of CDDP and 2) a significant decrease in cell viability must be observed in the presence of low-dose CDDP. The lethality of the knockdown termed “survival index” here, is determined based on cell counts relative to the negative controls within the same plate: a survival index of 100% suggests that gene knockdown has no effect on cell viability. The extent of potentiation is determined by the difference in cell count in the absence versus the presence of CDDP (IC_{10}), normalized to the BRCA2 positive control. The two parameters were combined to calculate a “gene score” to rank all genes. Genes with a high “gene score” and a high survival index (quadrant II, Figure 3.1A) would satisfy the selection criteria as CDDP activity enhancers. Since the WGS provided a biological replicate of the PKS, the two kinase datasets were analyzed independently to evaluate the reproducibility of our siRNA screen. The results are summarized in Figure 3.1A where each data point represents the results from one gene. The top 20 kinases from the PKS and WGS are highlighted in yellow crosses and red circles respectively. An overlap of 9 kinases in the two top-20 lists was observed (Figure 3.1A - red circles marked with X; Table A.4). Five of the top 20 kinases in WGS were not part of the PKS (green circles) as the WGS had 778 kinases in total. Using the same screening parameters, the 20 kinases with the strongest potentiation effects from the PKS were re-screened three times with a pool of three siRNA duplexes (Stealth siRNA) targeting each gene which were different than those used for the WGS and PKS. The Stealth siRNAs used were also chemically modified to increase the specificity and stability of the siRNAs. Here, PAPSS1 ranked consistently in all three independent experiments, as the top CDDP-potentiating candidate (Table A.5). The sensitization observed was further confirmed by repeating the screen using the three siRNA duplexes separately to ensure that the phenotype observed is not due to off-target effects (Figure A.3). Referring back to Figure 3.1A, PAPSS1 (crystal structure displayed in Figure 3.1B) ranked as the 7th and 18th kinase in the PKS and WGS respectively in contrast to its other isoform, PAPSS2, which ranked at ∼11,500 of 21,121 genes. When five of the top targets from the validation screen were further evaluated by generating CDDP dose response curves, PAPSS1 silencing demonstrated the most leftward shift in the dose response relative to the negative control scramble siRNA (Figure 3.1C). This was also reflected in the IC_{50} values for CDDP (Figure
3.1D). PAPSS1 inhibition when used in combination with CDDP appeared to sensitize A549 cells to an equal or greater extent compared to BRCA2 silencing (Figure 3.1B-C).

Figure 3.1. siRNA kinome screens identified PAPSS1 as a target that can be silenced to improve cisplatin activity. The results from the kinome screen are summarized in A, where each data point represents one gene being silenced in the presence or absence of CDDP. The x-axis indicates cell viability under gene-silencing condition in the absence of CDDP. The Gene Score on the y-axis is calculated as the product of survival index (cell viability from gene knockdown alone) and potentiation effects (the difference in cell count in the absence versus the presence of CDDP when the gene is silenced). The crystal structure of PAPSS1 is shown in B (PDB 1X6V) with the two enzymatic domains labelled. CDDP dose response curves (72 h treatment) were generated to further evaluate the top five targets (C). The data are plotted as fraction affected (mean ± SD) where a Fa value of 0 would indicate equivalent viable cell count in the treated well relative untreated controls. The IC$_{50}$ values interpolated from these fitted curves (mean ± 95% confidence intervals) are displayed in D. PAPSS1 silencing caused the most reduction in IC$_{50}$ relative to scramble (*p < 0.05) and improved CDDP response to an equal or a greater extent compared to BRCA2 silencing.

Further validation studies associating PAPSS1 silencing with enhanced CDDP activity in A549 cells are summarized in Figure 3.2. Under conditions where siRNAs strongly suppressed PAPSS1 mRNA and protein levels (Figure 3.2A-B), a significant shift in the CDDP dose response curve was observed (Figure 3.2C). Note that the loss of PAPSS1
expression did not affect PAPSS2 expression (Figure 3.2C). PAPSS1 knockdown led to a 5.4-fold and 6.8-fold decrease in the CDDP IC₅₀ relative to untransfected and scramble controls, respectively. The potent combinatorial effects of PAPSS1 inhibition and CDDP are apparent in representative images shown in Figure 3.2D. Although PAPSS1 silencing had little impact on cell viability in the absence of CDDP, the images (Figure 3.2D) suggested that silencing alone engendered changes in cell morphology. To explore this further, the long-term effects of PAPSS1 inhibition were investigated using a clonogenic assay. As shown in the representative images in Figure 3.2E supported by a quantitative assessment of plating efficiency (Figure 3.2F), PAPSS1 knockdown alone significantly decreased the clonogenicity of A549 cells when compared to cells transfected with the scramble siRNA. More importantly, PAPSS1 inhibition in combination with low-dose CDDP (IC₅₀) reduced the plating efficiency by about 98.7% relative to scramble controls (p<0.001; Figure 3.2E and F).
Figure 3.2. Validation of siRNA screen in A549 cells. Using PAPSS1-targeting siRNA duplexes, PAPSS1 expression was reduced by > 90% at the mRNA level (A; ** p < 0.01). This knockdown was confirmed by Western blot analysis (B; 89.31% knockdown). PAPSS1 silencing (solid squares) yielded a leftward shift in the CDDP dose response curve relative to controls (C). The representative fluorescent (upper panels) and bright field (lower panels) images at selected doses of CDDP in cells transfected with scramble or PAPSS1 siRNA obtained with IN Cell Analyzer are shown (D; 10x magnification). Cell viability was assessed based on detection of plasma membrane integrity 72 hours following CDDP treatment. Total and dead cell counts are determined using Hoechst 33342 (blue) and ethidium homodimer (red) staining. The long-term effects of PAPSS1 knockdown with and without CDDP were explored using clonogenic assays. Representative images of each treatment condition are shown (E). The plating efficiency, defined as the number of colonies formed from the number of trypan blue excluding cells is plotted as means ± SEM (F; ***p < 0.001).
3.3.2 PAPSS1 Silencing Potentiates Cisplatin Activity in a Dose-Dependent Manner

To assess how the level of PAPSS1 inhibition influenced CDDP activity, A549 cells were transfected with increasing concentrations of PAPSS1 siRNA and PAPSS1 protein expression was determined by Western blot analysis (Figure 3.3). As shown in Figure 3.3A, there was an siRNA dose-dependent decrease in PAPSS1 protein levels. Next, CDDP dose response curves (DRC) were performed in A549 cells exposed to decreasing amounts of PAPSS1 siRNA (Figure 3.3B). At the highest siRNA dose used (25nM), the greatest shift in the CDDP DRC was observed with no significant change in the CDDP DRC at 3.125 nM of siRNA. A densitometry-based plot of fold-decrease in CDDP IC<sub>50</sub> versus protein expression level demonstrates a correlation between reduction in PAPSS1 protein level and increases in CDDP activity (Figure 3.3C); increases in CDDP activity were greatest when PAPSS1 silencing was >75%.

Figure 3.3. Cisplatin potentiation is dependent on the level of PAPSS1 silencing. Western blot showing the siRNA dose-dependent reduction in PAPSS1 protein expression (A). The correlation between the extent of CDDP activity enhancement and siRNA concentration is demonstrated in the differing levels of leftward shift in the CDDP dose response curve (B; mean ± SEM). The fold-change in the CDDP IC<sub>50</sub> was plotted against the band intensity from the western blot (C; error bars represent 95% confidence intervals). PAPSS1 expression was determined by normalizing PAPSS1 band intensity to β-Actin band intensity. The calculated value for each dose was then normalized such that 100% would be equivalent to untransfected (0 μM) control.
3.3.3 PAPSS1 Silencing Potentiates CDDP Activity in NSCLC Cell Lines with Different Genetic Backgrounds but Does Not Increase Cisplatin Cytotoxicity in Normal Lung Epithelial Cells

To exclude a possibility that enhancement of CDDP activity with PAPSS1 silencing observed in A549 cells is a cell line specific event, cytotoxicity curves were produced using H358, H1703, and H460 NSCLC cells. These cell lines, including A549, all harbor wild-type EGFR but differ in their tumor subtype and p53 and KRAS mutational status (Table A.6) [180]. The cytotoxicity data show that PAPSS1 silencing results in 1.8, 3.3, and 6.5-fold decrease in IC_{50} in H358, H1703, and H460 cells, respectively (Figure 3.4A) when significant PAPSS1 silencing was achieved at the protein level (Figure 3.4B). Although H460 was most sensitized to CDDP treatment when PAPSS1 expression was inhibited, it was also most sensitive to PAPSS1 knockdown, with approximately 40% loss in cell viability within 96 hours in the absence of CDDP (Figure A.5). Since PAPSS1 silencing appeared to be less lethal in A549 cells while still engendering a 5-fold enhancement in CDDP activity, A549 was chosen for further studies.

Figure 3.4. PAPSS1 silencing enhances cisplatin activity in NSCLC cell lines with different genetic background. PAPSS1 was silenced using siRNA methods in a number of different non-small cell lung cancer cell lines (A). CDDP cytotoxicity in scramble or PAPSS1 transfected cells (B). Data are plotted as mean ± SEM, representative of at least three independent experiments; **p < 0.05 relative to scramble control.
To investigate how PAPSS1 silencing affects normal lung cells, human lung microvascular endothelial cells (HLMVEC) and human bronchial epithelial (HBEp) cells were grown to complete confluence to model non-proliferating normal tissue and then transfected with PAPSS1-targeting siRNA (or scramble control) followed by addition of CDDP 24 hours later. In HLMVEC, substantial PAPSS1 knockdown could not be achieved (<70% reduction at the messenger RNA level and little PAPSS1 loss at the protein level) even when using the highest siRNA concentrations possible (Figure A.5). Consistent with the data in Figure 3.3, the PAPSS1 suppression in this primary cell line was not sufficient to determine a change in the CDDP dose response curve. The lack of ability to induce strong inhibition of PAPSS1 in the endothelial cells is likely due to its inherently high level of expression in these cells [181]. In HBEp cells, substantial knockdown of PAPSS1 was achieved at the protein level (Figure 3.5A) with >90% reduction in mRNA expression (data not shown). Over the period of 72 hours following transfection, viability of confluent HBEp cells did not decrease. Importantly, there was no significant difference in CDDP activity observed in PAPSS1-silenced cells (Figure 3.5B). Similar CDDP-induced cytotoxicity was observed in control and PAPSS1-silenced HBEp cells at high (>10 µM) CDDP concentrations. HBEp cells transfected with PAPSS1 or scramble siRNA were also subjected to cell cycle analysis following treatment with an effective dose of CDDP (22 µM) for 72 hours. As shown in Figure 3.5C, unlike the NSCLC line (see Section 3.3.4 below), PAPSS1 silencing did not affect cell cycle distribution or the fraction of apoptotic cells in the normal epithelial cell population.
Figure 3.5. PAPSS1 silencing does not enhance cisplatin activity in HBEpC normal airway cells. Gene knockdown was confirmed by Western blot analysis at 72 hours post-transfection (A). Data from three CDDP dose response curves were averaged and plotted as mean ± SEM (B). HBEpC cells were also subjected to cell cycle analysis by flow cytometry (C; apoptotic populations are marked with a horizontal marker).

3.3.4 PAPSS1-Silencing in Combination with Cisplatin Increases DNA Damage and Induces G1/S Phase Arrest and Apoptosis

To gain a better understanding of the mechanism by which PAPSS1 sensitizes A549 cells to CDDP treatment, the effects of gene knockdown on cell cycle and the levels of sub G0/G1 population, considered apoptotic, were measured by flow cytometry at 24 (Figure 3.6A) and 48 hours (Figure 3.6B) following treatment. Quantification of the cell cycle distribution and apoptotic fraction can be found in Figure A.6. The data demonstrate that at both 24 and 48 hours, a G2/M block (blue arrows) was observed at the highest CDDP dose tested in the scramble control while a marked G1/S block (red arrowheads) was observed in PAPSS1-silenced cells treated with CDDP (Figure 3.6A and B). In addition, in the presence of CDDP, a small increase in the population of sub G0/G1 apoptotic cells is noticeable at 24 hours in PAPSS1-silenced cells compared to scramble controls (Figure 3.6A) and this difference becomes very prominent at 48 hours post-treatment (Figure 3.6B). Consistent with the flow cytometric data, Western blot analysis shows that PAPSS1 silencing in the presence of CDDP results in increased expression of the common apoptotic...
markers cleaved caspase-3 and cleaved PARP (Figure 3.6C). Consistent with the accumulation of cells in the G1/S phase, there is also a significant up-regulation of cyclin E and down-regulation of cyclin A expression when PAPSS1 knockdown was combined with CDDP (Figure 3.6C).

**Figure 3.6.** PAPSS1 knockdown in combination with cisplatin induces G1/S-phase arrest and produces high rates of apoptosis. A549 cells were treated with selected concentrations of CDDP for 24 (A) or 48 hours (B). 24 hours following transfection and the effects of both PAPSS1 silencing and CDDP treatment on cell cycle distribution are summarized here. The apoptotic population is marked with a horizontal line. The blue arrow points to the G2/M phase block in the cell cycle while the red arrow heads indicate a G1/S phase block. Western blot analyses of the expression of cyclins and common apoptotic markers (cleaved PARP and cleaved caspase-3) are shown (C) in A549 cells that were transfected with scramble or PAPSS1 siRNA and then treated with 0.71 μM (IC10) of CDDP for 24 hours.

Based on these data, we hypothesized that PAPSS1 activity may normally be involved in mitigating CDDP induced DNA damage or in promoting DNA repair following CDDP treatment. To date, the most sensitive biomarker used to assess the DNA damage that
correlates with DNA strand-breaks is phosphorylation of histone H2AX at Ser 139 (γH2AX) [182]. Thus, flow cytometry was performed to measure the levels of γH2AX under PAPSS1-silencing conditions (Figure 3.7A-B). As shown in Figure 3.7A, low-dose CDDP caused a slight increase in γH2AX in scramble controls. However, when PAPSS1 was silenced, a marked increase in γH2AX was observed. The levels of γH2AX at each tested dose are shown in the corresponding plots (Figure 3.7B). PAPSS1-silencing resulted in significantly more γH2AX-labeling in the presence of low doses of CDDP (0.1 and 0.5 µM). Since topotecan, a commonly used positive control for γH2AX, is also a DNA damaging agent, we used low doses of topotecan (20 and 40 nM) to see whether the increase in γH2AX levels could be observed with a non-platinum DNA damaging agent. As shown in Figure 3.7A-B, PAPSS1 knockdown in combination with low doses of topotecan resulted in greater accumulation of γH2AX. Interestingly, PAPSS1 knockdown alone appears to induce DNA damage, albeit at low levels, in both CDDP and topotecan treated cells. These results were further confirmed using immunofluorescence staining for γH2AX and high content analysis (HCA) (Figure 3.7C-D). Figure 3.7C shows the representative images of γH2AX foci in scramble and PAPSS1 siRNA-transfected cells treated with low doses of CDDP or topotecan. At each dose, significantly more cells with γH2AX-positive puncta were detected in PAPSS1 silences cells. Further, PAPSS1-silenced cells had more cells with multiple γH2AX foci, which are less likely to survive (Figure 3.7D). These data were consistent with the flow analysis shown in Figure 3.7A and B confirming that γH2AX expression is enhanced when PAPSS1-silenced cells are treated with low doses of CDDP or topotecan. These data suggest that PAPSS1, when expressed at normal levels, is involved in reducing the amount of DNA damage caused by CDDP and topotecan, either by blocking drug action or promoting efficient DNA repair.
Figure 3.7. Relative to non-silenced controls, more DNA damage occurs when PAPSS1-silenced A549 cells are treated with cisplatin or topotecan. The representative histograms illustrating the expression of γH2AX are shown (A). Topotecan (250 nM for 24 hours) was used as a positive control to set up gating parameters. The quantified γH2AX population at each dose of cisplatin (CDDP) and topotecan (TPT) under PAPSS1-silencing and non-silencing conditions are plotted (B). For immunofluorescent staining, cells were treated with CDDP or TPT, stained with anti-γH2AX antibody (red) and counterstained with Hoechst 33342 (blue) (C). Images were visualized using the IN Cell Analyzer 2200 (20X/0.45 objective). Percentage of cells with more than one γH2AX-positive puncta are quantified and plotted in D. All data are displayed as mean ± SD; **p < 0.01, ***p < 0.001.
3.3.5  

**MET3 and MET14 Deletion Do Not Sensitize Yeast to Cisplatin Treatment**

In attempt to gain a better understanding of how PAPSS1 silencing affects increases in CDDP activity, we initiated studies in *Saccharomyces cerevisiae* by generating double mutants and performing spot and CDDP cytotoxicity assays [183]. The ATP sulfurylase and APS kinase domains that make up PAPSS enzymes are encoded by two separate genes in *Saccharomyces cerevisiae*, met3 and met14 respectively. In yeast, the metabolite PAPS plays a role in amino acid metabolism but unlike humans, yeast do not use sulfotransferases to detoxify chemicals nor do they modify proteins by sulfonation. We found that single and double deletion mutants of the yeast MET3 and MET14 genes were not more sensitive to CDDP than an isogenic wildtype strain (see Figure A.7). These results suggest that the phenotype observed with PAPSS1 inhibition and low-dose CDDP in cell lines stems from a PAPSS function unique to those cells.

3.3.6  

**PAPSS1 Silencing Sensitizes NSCLC Cells to Radiation and Treatments with DNA Crosslinkers and Topoisomerase I Inhibitors**

Thus far the results have focused on CDDP and we have clearly shown that in PAPSS1-silenced cells, low doses of CDDP increase the number of cells arresting in the G1/S phase (Figure 3.6) and increase γH2AX-labeling (Figure 3.7): effects that are associated with a significant increase in CDDP cytotoxicity even at low doses of CDDP. Since PAPSS1 knockdown also appears to increase DSBs in cells treated with topotecan, we speculated that the mechanisms linked to PAPSS1 silencing-induced increases in CDDP activity are not CDDP-specific. To test this hypothesis, we evaluated how PAPSS1-silencing influenced the activity of other DNA damaging agents. The data presented in Figure 3.8 show that PAPSS1 knockdown sensitized A549 cells to radiation (p<0.01), reducing the surviving fraction by up to 7-fold at 8 Gy relative to scramble controls. PAPSS1 silencing also enhanced the activity of other DNA crosslinkers, such as carboplatin, oxaliplatin, mitomycin C, as well as the selected topoisomerase I inhibitors, causing at least a 2-fold decrease in the IC50 of these agents (Table 3.1). On the other hand, no changes in cytotoxicity were observed when A549 cells were treated with the selected mitotic and topoisomerase II inhibitors.
These results indicate conclusively that the mechanism of PAPSS1-silencing induced enhancement of CDDP activity is not CDDP-specific and therefore is not directly related to the role of sulfotransferases in CDDP metabolism. Further, the results indicate that targeting PAPSS1 has the potential to potentiate many broad-spectrum genotoxic therapies; very likely through mechanisms that involve increased DNA damage or reductions in DNA repair.

Figure 3.8. PAPSS1 silencing sensitizes A549 cells to radiation, as well as platinum-based agents and topoisomerase I inhibitors. Cells transfected with PAPSS1 or scramble siRNA were subjected to selected doses of radiation. Data were normalized to non-irradiated controls (mean ± SD; n = 3). The dose response curves from chemotherapeutic treatments are displayed as mean ± SEM and are representative of at least three independent experiments. Statistical analysis was performed on the radiation study using the SPSS statistical software. The scramble and PAPSS1 dose response curves following radiation are significantly different (p<0.01).
Table 3.1. IC_{50} Values of Chemotherapeutics in A549 Cells

<table>
<thead>
<tr>
<th>Chemotherapeutic Agent</th>
<th>Scramble</th>
<th>PAPSS1 Knockdown</th>
<th>Fold-Change in IC_{50}</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mitotic Inhibitors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vincristine</td>
<td>29.0 nM</td>
<td>32.0 nM</td>
<td>0.906</td>
<td>ns</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>0.54 µM</td>
<td>0.61 µM</td>
<td>0.885</td>
<td>ns</td>
</tr>
<tr>
<td><strong>DNA Crosslinkers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>2.44 µM</td>
<td>0.44 µM</td>
<td>5.55</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>Carboplatin</td>
<td>30.0 µM</td>
<td>10.2 µM</td>
<td>2.94</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>3.31 µM</td>
<td>1.41 µM</td>
<td>2.35</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>68.2 nM</td>
<td>16.5 nM</td>
<td>4.13</td>
<td></td>
</tr>
<tr>
<td><strong>Topoisomerase I Inhibitors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Topotecan</td>
<td>78.9 nM</td>
<td>37.8 nM</td>
<td>2.09</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>Irinotecan</td>
<td>9.7 µM</td>
<td>4.3 µM</td>
<td>2.26</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td><strong>Topoisomerase II Inhibitors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>83.5 nM</td>
<td>71.9 nM</td>
<td>1.16</td>
<td>ns</td>
</tr>
<tr>
<td>Epirubicin</td>
<td>69.0 nM</td>
<td>51.9 nM</td>
<td>1.33</td>
<td>ns</td>
</tr>
</tbody>
</table>

*ns = IC_{50} values between control and PAPSS1-silenced cells are not statistically significant*

### 3.4 Discussion

The typical path of developing combinations for the treatment of any cancer has been combining new drugs with “standard” drugs that are known to provide some clinical benefits. For NSCLC patients, this defines a 30-year history of clinical trials combining CDDP with the next best drug with no meaningful gain in overall survival [184, 185]. Alternatively, approaches designed to enhance CDDP activity in first-line therapy may provide therapeutic benefits to this patient population. Here, we applied the siRNA screening approach to identify gene targets that would enhance the cytotoxic effects of low-dose (IC_{10}) CDDP in chemo-naïve NSCLC cells. Utilizing a sub-lethal dose of CDDP considers the fact that in solid NSCLC tumors, cells are exposed to a range of CDDP concentrations depending on tumor architecture and microenvironment. Cancer cells exposed to high (cytotoxic) drug levels will die whereas those exposed to sub-lethal drug doses develop cytoprotective responses that promote survival. We demonstrated here, for the first time, that PAPSS1 silencing can enhance CDDP activity in multiple NSCLC cell lines. Note that
silencing of PIP4K2A, the top kinase from both the PKS and WGS which has also been recently identified as a pharmaceutical target in p53-mutated cancers [186], yielded only a modest shift in the CDDP dose response curve compared to PAPSS1 in A549 cells (Figure 3.1B-C). The increase in CDDP activity seen when PAPSS1 was silenced was comparable to that observed when BRCA2 was silenced. Although it is difficult to translate the changes observed in vitro to a clinical setting, BRCA2 deficiency is known to be associated with better treatment response to platinum drugs in patients [114, 187-189].

Regarding the clinical relevance of PAPSS1 it is important to recognize that this protein is expressed in normal and cancer cells alike and based on the data shown in Figure 3.5C, we can conclude that sensitization to CDDP would only be observed in cell populations where PAPSS1 expression is very low. At this time there is a lack of gene expression data derived from lung cancer patients which would suggest that PAPSS1 levels are important in terms of predicting response to CDDP-containing cocktails. We are also not aware of any database that would provide these data in the context of CDDP treatment response. There are many research teams that are doing genomic analysis of lung cancer; however, the data that they are collecting on what type of treatments the patients received or how they responded to those treatments are scanty. There is a NCIC trial of adjuvant therapy in which 50% of patients receive adjuvant CDDP-vinorelbine and this trial has been able to accrue patients willing to provide tissue. To date, they have tissue from a few hundred patients with the goal of finding markers predictive of sensitivity. Further, these data are being collected in an adjuvant setting so results will not be linked to initial response - just time to recurrence and death. It should be noted that we have also searched bioinformatics databases, such as that held by the European Bioinformatics Institute (EBI, http://www.ebi.ac.uk/Information) which includes data on PAPSS1 mRNA expression obtained from 340 experiments representing 95 different disease states. These data would suggest that PAPSS1 is not differentially expressed in lung cancer cell lines. We have also searched the cBioPortal database which indicates that 6.9% of breast cancer patient xenografts have PAPSS1 amplification and about 2% of lung adenocarcinomas have PAPSS1 mutations. None of these existing datasets, however, link PAPSS1 expression levels to treatment responses to CDDP.
In terms of our screen results, it is worth noting that several other siRNA screens have been conducted to identify CDDP modulators in human cell lines, but none of these focused on lung cancer cells exposed to low-dose CDDP. Table A.7 displays our screen results for 30 genes that were previously identified by other groups as CDDP modulators. Swanton et al. performed an RNAi screen with 779 kinases to identify modulators of paclitaxel and CDDP in A549 and two other non-NSCLC cell lines [98]. Even though our experimental conditions, endpoint assay, and method of data analysis differ, our results have considerable overlap with those described by Swanton and colleagues. For instance, STK16, a serine/threonine protein kinase that sensitized the cell lines to paclitaxel and CDDP treatment in the Swanton et al. study [98], was also identified as one of the top CDDP enhancers in our screen (Table A.4 and Table A.7). Similarly, CDK5R1 silencing potentiated CDDP activity in our screen as well as that performed by Swanton et al. (Table A.7). In our screen, CDK5R1 silencing was associated with a 23% loss in cell viability in the absence of CDDP and a further 40% reduction in cell count in the presence of CDDP. Although considered as a “hit”, the loss of cell viability with CDK5R1 knockdown alone would lower its ranking in our screen. It should be noted that PAPSS1 was not included in the screen completed by Swanton et al.

RNAi screens for CDDP modulators were also performed in cervical malignant HeLa cells, SKOV3 ovarian carcinoma cells, and BJET-p53KD immortalized human fibroblast cells [92, 114, 135]. Bartz et al. identified multiple genes involved in DNA damage repair (BRCA1, BRCA2, and REV3L) as CDDP potentiators in HeLa cells which were further supported by similar results obtained by Nijwening et al. in fibroblast cells [114, 135]. These same genes also significantly enhanced CDDP activity in our screens but when these genes were silenced in the absence of CDDP, approximately 30% reduction in cell viability was observed. For this reason, these “hits” were not included in our initial data assessment. It is worth noting that one of the top protein kinases identified in our screen was RPS6KA3 (Table A.4), which codes for RSK2, a protein kinase involved in cell cycle progression when the Ras-ERK pathway is activated. This particular target has recently been validated as a CDDP activity enhancer in ovarian cancer cells [190]. Our rich dataset along with our stringent target selection approach therefore provide comparable results to previously
published screens while also uncovering previously unrecognized CDDP enhancers such as PAPSS1.

Given the biological role of PAPSS1 [179, 191-193], one can speculate on the role of sulfur metabolism and homeostasis in cancer cells when they are first exposed to cytotoxic agents such as CDDP. Previous studies have shown that low methionine diets could reduce the concentration of inorganic sulfate in the serum of rats and the hepatic concentration of PAPS [194]. It is also known that many cancer cells, but not normal cells, are methionine-dependent [195, 196]. However, our analysis of yeast deleted for the genes encoding ATP sulfurylase and APS kinase reveal that neither activity in amino acid metabolism is important for CDDP sensitivity (Figure A.7). These results support a model in which PAPSS1 knockdown-induced sensitization to DNA damaging agents in cancer cell lines is related to an evolutionarily specialized role of sulfonation, rather than the conserved role in amino acid metabolism that is shared with yeasts. Furthermore, both MET3 and MET14 localize to the cytoplasm of yeasts [197], consistent with the distribution of PAPSS2 in humans [192]; whereas, human PAPSS1 localizes to the nucleus, suggesting that sulfonation plays important roles within the nucleus of human cells that might be responsible for the phenotype being observed in our studies. Based on the results presented here, sulfonation reactions may play a more important role in the survival of the cancer cell population than previously recognized. PAPS is the required source of biological sulfate for all sulfotransferases [198] which are involved in second phase metabolism of xenobiotics. Therefore, one could postulate that PAPSS1 inhibition may affect the availability of thiol-containing compounds and the metabolism of CDDP and other anticancer agents [176, 198, 199]. It is known that mutations in sulotransferase 1A1 (SULT1A1) are associated with increased lung cancer risk, especially for cigarette smokers [200]. Silencing of SULT1A1 and several other sulfotransferases also appear to enhance CDDP activity in our WGS (data not shown), albeit not to the same extent seen when PAPSS1 was silenced. Although sulfonation likely plays a role in CDDP metabolism, the effects of PAPSS1 silencing on increasing the activity of radiation, mitomycin C and topoisomerase I inhibitors cannot be explained by the role of sulfotransferases in drug metabolism.
Thus far, our yeast data and the broad-spectrum sensitization to DNA-targeting chemotherapeutics and radiation observed have ruled out the two mechanisms of action: sulfate usage for amino acid metabolism and detoxification of xenobiotics. Our third speculated mechanism of action involves post-translational sulfonation of proteins. However, with very little known about PAPSS1 in the literature and the numerous roles that sulfonation plays in cell biology, identification of sulfonated protein(s) responsible for the observed sensitizations is beyond the scope of this study. The exact mechanism by which PAPSS1 enhances the activity of multiple cytotoxic agents will take time to fully elucidate, particularly since the cellular responses to genotoxic agents, CDDP for example, are still not completely understood even though these agents continue to form the mainstay of chemotherapy for patients with cancer [201]. We believe that the flow cytometric and colony formation assay data suggest that the lack of PAPSS1 function alone can induce apoptosis, leading to accumulation of some DNA damage as detected by γH2AX staining, and reduce the survival of A549 cells. Note that not all cells were γH2AX-positive and some cells had dim staining. This is likely due to the fact that the cells were not synchronized, resulting in differential staining and DNA damage effects depending on the stage at which each cell was in the cell cycle [202]. Altogether, data actually suggest that the effects achieved when CDDP is combined with PAPSS1 silencing are highly synergistic [15]. Consistent with our results (Figure 3.6), cells without PAPSS1 suppression arrest at the G2/M transition when treated with CDDP [203] and may remain in this phase for days before committing to apoptosis or surviving by progressing through the cell cycle [203]. However, instead of seeing a further increase in the accumulation of cells at the G2/M transition, cells lacking PAPSS1 expression in the presence of CDDP accumulated in the G1/S phase. This was associated with upregulation of cyclin E which controls the G1/S transition and downregulation of cyclin A, which is responsible for S/G2 progression [204, 205]. It could be speculated that the cells are progressing to G1/S phase to initiate DNA replication but fail to progress further into late S and G2/M phases of the cell cycle [205]. The amount of DNA damage induced by CDDP and topotecan was quantified by measuring γH2AX (Figure 3.7), which is formed in the presence of DNA strand breaks to recruit DNA repair proteins [182]. With CDDP and topotecan, more DNA damage was detected when cells had reduced PAPSS1 expression. More important, there is significantly more cells with multiple γH2AX foci in PAPSS1-silenced cells at all doses and it is reasonable to assume that
cells with multiple γH2AX foci are more likely to commit to cell death. Altogether, these results suggest greater accumulation of DNA damage when PAPSS1 expression is low and this could be due to either increased DNA damage or reduced rates of DNA repair. Whether the reduction in PAPSS1-mediated sulfonation is indirectly responsible for impairment of DNA repair mechanisms or is an autonomous mechanism contributing to the synergy with CDDP remains to be established.

Although PAPSS1 silencing sensitized A549 cells to platinum-based agents as well as the non-platinum DNA crosslinker mitomycin C, topoisomerase I inhibitors, and radiation, silencing PAPSS1 did not sensitize A549 cells to topoisomerase II inhibitors. A recent publication by Maede et al. demonstrates that topoisomerase I and topoisomerase II inhibitors induce different types of DNA lesions, which are in turn repaired by different pathways [206]. Similarly, Cummings et al. reported that suppression of ERCC1, a gene involved in both nucleotide excision repair (NER) and HR, sensitizes prostate cancer cells to both mitomycin C and CDDP while inhibition of XPA, which is only involved in NER, only sensitizes the cells to CDDP [207]. The results we presented here suggest that PAPSS1 silencing may be associated with impairment of particular DNA repair mechanism(s) that consequently sensitize cancer cells to specific anticancer agents depending on the DNA lesions induced and the cellular processes that are important for repairing those lesions.

The data reported in Figures 3.3 and 3.4 suggest that strong PAPSS1 inhibition enhances CDDP activity in four different NSCLC cell lines and these data stress the importance of identifying small molecule inhibitors of PAPSS1 as siRNA therapeutic approaches will likely be difficult to deliver in therapeutically relevant doses to achieve sufficient inhibition of PAPSS1 within a heterogeneous population of tumor cells. In this regard, we tested chlorate, a PAPSS inhibitor that inhibits the first step of PAPS synthesis by blocking ATP sulfurylase activity [208]. When cells were pretreated with a non-toxic dose of chlorate (50 mM), the CDDP IC₅₀ reduced by about 2-fold (Figure A.8). Chlorate has been used by multiple groups to inhibit sulfonation [208, 209] and previous reports have shown that chlorate can halt cell cycle progression at the S phase [210], a result similar to that observed in our study in PAPSS1-silenced cells treated with CDDP (Figure 3.6). We have not pursued these studies further because chlorate is a non-isoform-specific inhibitor that is active only when added at very high concentrations. This agent cannot be pursued as a
therapeutic compound against PAPSS1 and it is not appropriate as a tool compound to help us understand the mechanisms through which PAPSS1 inhibition causes increased sensitivity to DNA damaging agents. Moving forward, a small molecular inhibitor screen would be a rational approach to identify and validate potent PAPSS1 inhibitors for use as a therapeutic agent. This interaction between PAPSS1 silencing and enhancing the activity of several distinct classes of commonly used anticancer agents is unique and is worth pursuing therapeutically in cancers where these drugs are commonly used.
4. In Vivo Validation of PAPSS1 (3’-phosphoadenosine 5’-phosphosulfate synthase 1) as a Cisplatin-Sensitizing Therapeutic Target

4.1 Introduction

We have reported in the previous chapter on an siRNA screen which identified 3’-phosphoadenosine 5’-phosphosulfate (PAPS) synthase 1 (PAPSS1) as a novel therapeutic target that can be suppressed to enhance the activity of multiple DNA damaging agents including the platins, causing increased accumulation of DNA damage ultimately leading to apoptosis [97]. PAPSS1 is an enzyme that converts inorganic sulfate to the biologically active form PAPS which is then used for cellular sulfonation reactions [211, 212]. We have validated that PAPSS1 knockdown sensitizes four different NSCLC cell lines to cisplatin (CDDP) treatment with no specificity to histological subtype or driver mutation (see Section 3.3.3). We also confirmed that the potentiation effects were cancer-specific as PAPSS1 knockdown had no effect on CDDP toxicity in non-proliferating normal human bronchial epithelial cells. The original goal of the siRNA screen was to identify hits that would enhance the activity of low-dose CDDP such that cells that are normally exposed to sub-lethal concentrations of CDDP will commit to cell death. It is, however, important to recognize that tumour cells that are exposed to low doses of chemotherapeutics often also suffer from restricted access to oxygen and nutrients due to their large distances from blood vessels [213]. Modulators of CDDP or other drugs of interest should therefore be evaluated in relevant models that take these variables into consideration. In this chapter, we first assessed the therapeutic value of targeting PAPSS1 as a CDDP modulator in vitro by testing the effects of PAPSS1 knockdown on CDDP activity in NSCLC cells subjected to serum starvation and chronic hypoxia. We then provide further support that PAPSS1 is a target that should be pursued to improve the efficacy of platinum-based agents in the first line setting through three different in vitro and in vivo model systems: spheroids, zebrafish xenografts, and shRNA-based xenografts in mice.
4.2 Methods

4.2.1 Cell Culture and Reagents

A549 cells were cultured as previously mentioned (see Section 3.2.1). A549 NucLight Red cells were purchased from Essen Bioscience and maintained in Ham’s F-12 Nutrient Mix with GlutaMAX medium (Life Technologies) supplemented with 10% FBS and 2 mM L-glutamine. Red fluorescence was sustained with 0.5 µg/mL puromycin (Clontech Laboratories) until the start of an experiment. PAPSS1 (1:1000) primary antibody was obtained from Abcam while β-Actin (1:50000) primary antibody was purchased from Cell Signaling Technology. CDDP was obtained as ready-to-inject solutions from Hospira.

4.2.2 siRNA Transfections

Cells were seeded at 200,000 cells/well in 6-well plates using OPTI-MEM medium (Life Technologies) supplemented with 4% FBS and 2 mM L-glutamine. The following day, a pool of three siRNA duplexes targeting PAPSS1 (HSS113394, HSS189820, HSS189821; Life Technologies) was complexed with the lipid-based transfect reagent RNAiMAX (Life Technologies) at a ratio of 170 µL lipid/nmol siRNA at room temperature for 20 minutes under serum-free conditions and then added to cells to achieve a final concentration of 75 nM siRNA. The Stealth RNAi negative control kit (Life Technologies) was used as scramble control.

4.2.3 Starvation Cell Viability Assays

Cells were reverse-transfected with siRNAs in 384-well plates. At 30 hours post-transfection, the media were replaced with serum-free culture media. The following day, the cells were treated with different concentrations of CDDP. At 48 and 72 hours following drug treatment, cytotoxicity was assessed using a fluorescence-based metabolic assay. PrestoBlue (Life Technologies) was used according to manufacturer’s instructions and the cells were incubated at 37°C for 1 hour. Metabolic activity was measured using FLUOstar OPTIMA microplate reader (BMG Labtech). All data were plotted using Prism 6.0 (GraphPad Software) as mean ± SEM from three independent experiments.
4.2.4 Hypoxia Studies

Cells were reverse-transfected as described above. At 30 hours post-transfection, the cells were transferred to an incubator inside hypoxic chamber (Coy Lab Products) where the oxygen level was maintained at 1.0%. The following day, drug dilutions were prepared with media equilibrated under hypoxic conditions. At 48 and 72 hours following drug treatment, cells were removed from the chamber for assessment of cell viability using the PrestoBlue reagent as described above.

4.2.5 Spheroid Assays

NucLight red A549 cells were plated and transfected in 6-well plates as described above. At 48 hours post-transfection, cells were harvested by trypsinization and then seeded in 96-well round bottom ultra-low attachment plates (Corning) at 2500 cells/well for spheroid formation. The plates were then centrifuged at 1000 rpm for 10 minutes at room temperature. To monitor spheroid formation, the cells were imaged every 3 hours using the IncuCyte ZOOM® Live Cell Imaging system (Essen BioScience). Three days later, the spheroids were treated with the indicated doses of CDDP. At this time, 62.5 nM SYTOX® Green nucleic acid stain (Life Technologies) was added as a dead cell marker. All spheroids were imaged every 3 hours for 8 days and the data were analyzed using the IncuCyte ZOOM 2015A software.

4.2.6 SDS-PAGE and Western Blot Analysis

All buffer chemicals were obtained from Sigma-Aldrich. Approximately 1 x 10^7 cells were lysed with lysis buffer (see Section 3.2.5 for lysis buffer reagents). Cellular lysates were subsequently clarified (20 min at 14,000 x g). Tumour samples were homogenized using FastPrep-24 (MP Biomedicals). Briefly, a piece of tumour (100-200 mg) was placed in a Lysing Matrix M tube containing a single 1/4” zirconium oxide-coated ceramic bead. Up to 1 mL of lysis buffer (described above) was added to cover the tumour and the samples were kept on ice for 5 minutes. Homogenization with the FastPrep instrument was performed at 6 m/s for 30 seconds for at least three cycles with 5-minute rests between cycles. After each run, the samples were centrifuged at 4°C at 1000 rpm for 20 seconds. The samples were homogenized until all the tissue was dissolved. The homogenates were
transferred to 1.5mL microcentrifuge tubes and centrifuged at 4°C at 14,000 rpm for 20 minutes. The lysates were then transferred to new microfuge tubes and clarified at 4°C at 14,000 rpm for 10 minutes. For all samples, protein concentrations were determined using the BCA Protein Assay Kit (Pierce). Lysates were loaded into gels, transferred to nitrocellulose membranes, and probed with antibodies as described in Methods 3.2.5. Protein quantification was performed using the ImageLab software.

### 4.2.7 Zebrafish Husbandry

The transparent casper strain[214] of zebrafish used in the current study was maintained according to standard protocols[215].

### 4.2.8 A549 Cell Staining and Injection and Screening of Zebrafish

For in vivo zebrafish studies, A549 cells with or without siRNA knockdown of PAPSS1 were stained with CM-DiI (red fluorescence, Invitrogen). Cells were grown to confluence and trypsinized with EDTA. Cells were then washed with RPMI 1640 (Gibco), transferred to 15 ml Falcon tubes and centrifuged for 5 min at 1200rpm. Cells were re-suspended at a concentration of 1x10^7 cells per mL in PBS (Gibco) with 5 μg/mL CM-DiI. The suspension was incubated for 5 min at 37°C, and then 15 min at 4°C. Cells were washed once in PBS, and suspended in RPMI for injection into embryos.

48 hpf naturally dechorionated casper embryos were used for the xenotransplantation of A549 cells. Embryos were anesthetized with 0.2 mg/mL tricaine (Sigma). A manual PLI-100 microinjector (Medical Systems Corp.) was used to load the cell suspension into a pulled capillary needle for embryo injection. Approximately 150-200 A549 cells were injected into the yolk sac of each embryo. Following injection, embryos were kept at 28°C for 30 minutes and at 35°C for the duration of the experiments. At 12-24 hr post-injection (hpi), embryos were screened for the presence of a fluorescent cell mass within the yolk site. Positive embryos were isolated for experiments.

### 4.2.9 Quantifying A549 Cell Proliferation in vivo Using the Zebrafish

To determine the rate of cellular proliferation in vivo, injected embryos were placed into groups of 15-20 embryos and euthanized by a Tricaine (1mg/ml) overdose at 24 hr post-
injection (hpi) and 72 hpi time intervals. Embryos were placed in a microtube of 1.5 ml of pre-warmed protease solution (0.25% trypsin, 1mM EDTA and 45 ml 1X PBS). To this mix, 54 μl of collagenase (Sigma-Aldrich) at 100 mg/ml was added. Embryos were incubated undisturbed for 10 min. Subsequently, embryo-dissociation mix was homogenized using a Pasteur pipette by passing the dissociating embryos through the pipette and then quickly forcing them out against the bottom of the microtube approximately 10 times, every 5 minutes over a duration of 45 min. Upon completion, 300μl of 100% FBS was added to the microtubes to end the enzymatic reactions occurring between the dissociation mix and the embryo suspension. The suspension was then centrifuged at 1200rpm for 5 minutes and the supernatant removed, leaving approximately 100μl of liquid containing the pelleted suspension content with the single cells produced by the dissociation assay. These cells were washed once in chilled 1X PBS and finally re-suspended in 10ul per embryo of PBS-FBS (5% FBS) solution for imaging. The dissociations were analyzed using the inverted Axio Observer Z1 microscope. Six 10μl drops of the suspension were added to a microscope slide to create a hanging bolus. Boluses were analyzed as a mosaic 2 X 3 grid. The mosaic capture program complied equally sized square composite images that represented the entire centre of the circular bolus. Following capture, all individual images from the mosaic were analyzed using a semi-automated macro (ImageJ computer software) where relative fluorescent cell numbers could be determined per group/embryo.

4.2.10 Live Microscopy of Zebrafish Embryos

Every 24 hr for ~7 days, 4-6 embryos per experimental group were imaged and analyzed for cellular interactions within the zebrafish embryonic microenvironment. An inverted Axio Observer Z1 microscope equipped with a Colibri LED light source (Carl Zeiss Microimaging Inc.) and an Axiocam Rev 3.0 CCD camera and Axiovision Rel 4.0 software (Carl Zeiss Microimaging Inc.) was used to screen, observe, and capture images of injected embryos.

4.2.11 Generation of shRNA-Modified Cell Lines

GIPZ lentiviral shRNA particles targeting human PAPSS1 (Target Sequences: GTCTGGACATGCTTCCTAA, ACAAGTTTCATATCACCTT, GATCGATTCTGAATATGAA) were obtained from Thermo Scientific. Transduction of A549 cells was completed using the GIPZ
lentiviral shRNA starter kit. Briefly, A549 cells were seeded at 50,000 cells/well in 24-well plates. On the following day, the medium was removed and viral particles were added at 40 MOI (multiplicity of infection) with either single or combinations of two or three shRNA sequences. Four hours following transduction, full medium was added to the cells. At 48 hours post-transduction, the medium was replaced with fresh medium containing 1.5 μg/mL puromycin. Cells expressing high levels of GFP were selected and isolated as single cells using FACS (fluorescence-activated cell sorting) at the Flow Cytometry Core Facility (Terry Fox Laboratory, BC Cancer Research Centre). The colonies were maintained in selective media and finally validated via Western blotting over multiple passages. A non-silencing control cell line was generated in parallel with the PAPSS1-silenced cells.

4.2.12 Murine Xenograft Models

All inoculated cell lines were tested mycoplasma-negative through IDEXX BioResearch. Solid tumour models were conducted in SCID-RAG2 mice (6-10 weeks old) obtained from the British Columbia Cancer Agency Joint Animal Facility breeding colony and maintained in a pathogen-free environment. Model development studies were conducted in female mice and the final efficacy study was conducted in male mice based on animal availability. The solid tumor model was conducted by implantation of 5x10^6 parental, Negative control shRNA, or PAPSS1 shRNA A549 cells subcutaneously into the flank in a volume of 100 μL. All cells were used at exponential growth phase, up to a maximum of 10 in vitro passages. At 7 days following cell inoculation, the mice were treated intravenously with 3 mg/kg CDDP every 4th day x 3 (days 7, 11, 15).

Mice were monitored daily, and body weight measurements and signs of stress (e.g., ruffled coat and lethargy) were used as indicators of toxicities. Tumour size was measured using an electronic caliper (Mitutoyo Corp.) and the tumour volumes were calculated using the equation (l x w^2)/2. Animals with ulcerated tumours or whose tumours exceeded 500 mm^3 were euthanized. All tumours were collected and snap frozen at -80°C for further analyses. As a general comment, all in vivo studies completed under an animal care protocol (A14-0290) approved by the Institutional Animal Care Committee (IACC). The IACC for studies conducted at the BC Cancer Agency (Vancouver Cancer Research Centre) Animal Resource
Center is operated by the University of British Columbia in accordance to the Canadian Council of Animal Care (CCAC).

### 4.2.13 Statistical Analyses

All data are plotted using the Prism 6.0 (GraphPad Software) as mean ± SEM unless otherwise stated. Two-way ANOVA followed by Sidak adjustments for multiple test comparisons was used to analyze changes in spheroid fluorescence area (size) and in A549 cell numbers in zebrafish embryos. Statistical significance of the differences in tumour volumes was determined by analyzing tumour size at the final time point using one-way ANOVA followed by Tukey adjustments for multiple test comparisons. The Log-Rank test was used to compare the Kaplan-Meier curves between the non-targeting shRNA control and the shPAPSS1 group. In all cases, an adjusted p-value < 0.05 was considered statistically significant.

### 4.3 Results

#### 4.3.1 PAPSS1 Knockdown Sensitizes NSCLC Cells to Cisplatin Treatment Under Stressed Conditions

Cancer cells that are exposed to low doses of chemotherapeutics are often localized at sites where there is limited access to oxygen and nutrients due to poor tumour vasculature. It is therefore important to address whether or not the potentiation effects observed with PAPSS1 knockdown are maintained when cells are deprived of oxygen and/or nutrients. Cells were conditioned to serum-starvation, chronic hypoxia (1.0%), or both prior to and throughout drug treatment. It is evident from Figure 4.1A that after 48 h of CDDP treatment, PAPSS1-silenced cells were more sensitive to CDDP treatment in all tested conditions. The potentiation effects observed in hypoxia are similar to that observed under normal tissue culture (21% oxygen, 10% serum) conditions. Under all tested stress conditions, the sensitization effects of PAPSS1 knockdown were greater compared to stress-free cells following 72 h of drug exposure as demonstrated by the greater leftward shifts in the CDDP dose response curves (Fig. 4.1B). This is further highlighted when comparing the averaged fold-reduction in IC_{50} values of PAPSS1-silenced cells relative to the scramble transfection control (Fig. 4.1C). The greatest potentiation effects were
observed when the cells were both serum-starved and exposed to low oxygen levels for 72 hours with a 50-fold reduction in the CDDP IC\textsubscript{50} (Fig. 4.1B-C) when PAPSS1 was silenced. Note that PAPSS1 knockdown alone, consistent with previous findings, did not induce significant lethality compared to the negative control in this 72-hour assay.

Figure 4.1. PAPSS1-silenced cells are more sensitive to cisplatin treatment when starved and/or exposed to low oxygen levels. Cells were reverse-transfected and then acclimatized overnight to the indicated stress conditions the following day. CDDP was subsequently diluted in the appropriate media and the cells were treated for 48 or 72h under stress. A normoxic, unstressed control was completed in parallel. The CDDP dose response curves for 48h (A) and 72h (B) are plotted as mean ± SEM from three independent experiments. The dose response curves were normalized to the no-CDDP control for each condition such that a fraction affected (Fa) of 1 represents 0% viability and Fa = 0 suggests 100% cell viability. The IC\textsubscript{50} values were interpolated and the fold-reduction in the IC\textsubscript{50} of the PAPSS1-silenced cells relative to the scramble control under each test condition are plotted with error bars representing 95% confidence intervals (C).

Note that PAPSS1 knockdown alone, consistent with previous findings, did not induce significant lethality compared to the negative control in this 72-hour assay.
### 4.3.2 PAPSS1-Silenced Cells Form Spheroids that are More Sensitive to Cisplatin Treatment

A549 cells transfected with PAPSS1-targeting siRNAs were able to form spheroids over a period of three days. Spheroid formation was reproducible and the spheroid sizes were comparable to the scramble control (approximately 350-400 µm) (*Figure 4.2*). Based on changes in fluorescence intensity during spheroid formation, it can be suggested that PAPSS1-silenced cells were proliferating at a slower rate (*Figure 4.2A*). The intensity of the red fluorescence is proportional to cell number. The spheroids were treated with low (1.56 µM) or high (12.5 µM) doses of CDDP and were left undisturbed for 8 days. At both treatment doses, PAPSS1-depleted spheroids were more susceptible to CDDP (*Figure 4.2B-C*); however, the differences in spheroid size at the high dose were not statistically significant when controls were compared to the PAPSS1-silenced spheroids. At the low dose of CDDP, PAPSS1-depleted spheroids were significantly more sensitive relative to the scramble control based on differences in spheroid size (*Figure 4.2B, p<0.0001*). Representative images of the spheroids under each condition are displayed in *Figure 4.2C* where the nuclei are labelled red and non-viable cells are labelled with the Sytox Green nucleic acid dye (see **Methods 4.2.5**). The change in spheroid size was not statistically different between the two types of spheroids when left untreated. Following addition of CDDP (1.56 µM final concentration), the control spheroids grew approximately 37% in size while the PAPSS1-silenced spheroids shrank by about 21% (*Figure 4.2C*) at 8 days. When exposed to 12.5 µM of CDDP, the spheroid size decreased by 31% and 46% for scramble and PAPSS1-silenced spheroids, respectively.
**Figure 4.2. PAPSS1-silenced cells form spheroids that are more sensitive to cisplatin treatment.** Cells with reduced PAPSS1 expression proliferate at a slower rate under conditions that encourage spheroid formation (A). Changes in spheroid size over an 8-day period following CDDP treatment are plotted (B). Representative images of the spheroids before treatment and at Day 8 following CDDP treatment are presented (C; red = viable cells, green = dead cells). All data are representative of three independent experiments and plotted as mean ± SEM.

### 4.3.3 PAPSS1 Silencing Sensitizes NSCLC Cells to Cisplatin Treatment in Zebrafish Xenografts

To confirm effects of PAPSS1 knockdown on A549 cell proliferation, a zebrafish xenotransplantation model pioneered in the Berman laboratory [216] was used. A549 cells (scramble control cells or PAPSS1-silenced cells) were injected into 2-day old zebrafish embryos (see Methods) and 1 day post-injection (dpi) live cell microscopy was used to
capture real time images of cell growth and proliferation in both CDDP treated and control (untreated) embryos. The results are summarized in Figure 4.3.

Injected embryos were dissociated at 2 dpi to determine baseline A549 cell numbers as a reference for comparison to later dissociation time points. At 4 dpi, a 1.93 fold-increase in cell number for both A549 scramble control and PAPSS1-silenced cells was observed in the absence of drug. This demonstrated that the cells survived and proliferated in vivo. Two doses of CDDP (0.125 mM and 0.25 mM) were utilized to determine the effects of PAPSS1 knockdown on A549 cell sensitivity to CDDP. Toxicity curves for CDDP in zebrafish were generated first, where non-xenotransplanted Casper zebrafish embryos were exposed to increasing doses of CDDP to determine the maximum tolerated dose (MTD). The MTD was defined as the dose where embryos survival was 80%. Half the MTD (MTD50) was determined to be 0.25 mM. CDDP-treated control and PAPSS1-silenced cells exhibited reduced cell proliferation in vivo, while sensitivity was measurably higher in PAPSS1-silenced cells (Figure 4.3B). At 0.125 mM CDDP, A549 with depleted PAPSS1 exhibited a 36% increase in cells whereas there was a 73% increase for A549 scramble control cells. When exposed to 0.25 mM CDDP, the proliferation of PAPSS1-silenced cells was inhibited, resulting in a 10% decrease in cell number when compared to pre-treatment levels. By contrast, A549 scramble cells displayed a 53% increase in cell number.
Figure 4.3. PAPSS1 silencing sensitizes NSCLC cells to cisplatin treatment in zebrafish xenografts. Zebrafish embryos were inoculated with scramble or PAPSS1 siRNA-transfected A549 cells and treated with different doses of CDDP. The mRNA and protein expression of PAPSS1 in the transfected cells were reduced by >90% and >80%, respectively. This level of knockdown is maintained for 7 days, which is longer than the duration of a typical zebrafish experiment. Representative bright-field and fluorescent images at 0 and 48 hours post-treatment (hpt) are shown for each condition (A). Untreated tumours doubled in cell number at 48 hpt (B). The xenografts were treated with a sub-lethal of CDDP (0.125 mM) which causes no significant change in cell number in the scramble control when compared to the untreated control as well as an effective (p = 0.0081) but non-curative dose (0.25 mM). CDDP treatment at both concentrations were effective against PAPSS1-silenced cells (p<0.005) compared to untreated controls. At both doses, PAPSS1-deficient cells were also more sensitive to CDDP treatment relative to the scramble controls (**p<0.01, **** p< 0.0001). All data are plotted as mean ± SEM (n=3).
4.3.4 PAPSS1 Knockdown Delays Growth of Tumours in Mice Treated with Cisplatin

A549 cells modified to express a PAPSS1-targeting shRNA were inoculated subcutaneously into mice. The growth rate of these cells *in vivo* is comparable to the parental cell line ([Fig. 4.4A](#)). An aliquot of the cells that was inoculated were assessed PAPSS1 expression via Western blotting (Day 0, [panel B and C](#)) and the same cell population was left in culture with no selective pressure and sampled again at 7 days post-inoculation for PAPSS1 protein expression (Day 7, [panel B and C](#)). PAPSS1 expression in this modified cell line was reduced by 50% and 46% (compared to scramble) on day 0 and day 7, respectively. However, PAPSS1 expression in tumours (300 – 400 mg) arising following injection of the shRNA-modified cells suggested that PAPSS1 protein levels were almost comparable to the control (shSCR cell line). Although this was a clonally selected cell line, it can be suggested from these data that there was a growth disadvantage for the cells with PAPSS1-targeting shRNA allowing for cells expressing normal levels to arise. Based on these results, the *in vivo* validation study was designed using a treatment schedule that started at a time when it was confirmed that PAPSS1 expression was low (~50%). CDDP treatment was initiated 7 days following cell inoculation using a Q4D x3 (every 4th day for 3 times) dosing schedule ([Figure 4.5A](#)), and the dose selected (3 mg/kg) was determined to be effective at slowing the rate of tumour growth in animals inoculated with the parental (A549) cells. Efficacy of the treatment was then measured based on changes in tumour volume and further delays in tumour growth. The results of the study, as presented in [Figure 4.5B-C](#), indicate that tumour growth following CDDP treatment was similar when comparing animals injected with the parental and the non-targeting shRNA control cells. The activity of CDDP (3 mg/kg given Q4D x3) was greater in mice injected with the cells modified with PAPSS1-targeting shRNA. Based on one-way ANOVA with Tukey adjustments, the mean tumour volume of the shPAPSS1 group on day 45 is significantly lower than both controls (adjusted p-values of 0.008 and 0.026 against parental and scramble respectively). To assess treatment efficacy, the time required for the tumours to reach 200 mm$^3$ was determined and plotted in [Figure 4.5C](#) as a Kaplan-Meier curve. This provides another view on the results demonstrating enhanced efficacy when cells with suppressed PAPSS1 expression are treated with a non-curative dose of CDDP (p = 0.0027 relative to scramble control).
Figure 4.4. PAPSS1 shRNA-modified A549 cells grow at comparable rates as the parental cells but regain PAPSS1 expression over time. Cells expressing PAPSS1-targeting shRNA grew slightly slower, but at comparable rates as the unmodified parental cell line in vivo (A; mean ± SEM). PAPSS1 expression of the cells implanted and the tumours harvested was assessed via Western blotting (B) and the quantified protein expression of the corresponding blots are plotted (C; KD levels = 50% on Day 0, 45.64% on Day 7, and 10.50% on Day >45).

Figure 4.5. A549 cells with reduced PAPSS1 expression are sensitized to cisplatin treatment in murine xenografts. A non-curative CDDP dose that demonstrates some therapeutic effects was chosen to validate PAPSS1 as a CDDP activity-enhancing target in a murine model (A; mean ± SEM; n=6). To assess the effects of PAPSS1 silencing in vivo, all mice were treated at 7 days following cell inoculation with 3 mg/kg CDDP (q4dx3). The tumour growth is displayed (B) with data plotted as mean ± SEM (n=5-8; *p<0.05, **p<0.01) and the time required for the tumours to reach 200 mm³ is
plotted as a Kaplan-Meier curve (C) and the Log-Rank test was applied to evaluate the statistical differences between the non-targeting shRNA control and the shPAPSS1 groups.

4.4 Discussion

We have demonstrated in Chapter 3 that NSCLC cells with reduced PAPSS1 (3’-phosphoadenosine 5’-phosphosulfate synthase 1) expression are more sensitive to CDDP treatments, studies that included several NSCLC cell lines of different genetic background and histological subtypes. This sensitization was not observed in normal bronchial epithelial cells. Two isoforms of PAPSS1 exist in humans: PAPSS1 and PAPSS2 [198, 217]. PAPSS1 and PAPSS2 share 76.5% amino acid sequence identity and most differences are in the intronic regions and exons 1 and 12 of the 12-exon protein [179, 192]. We have also reported in Chapter 3 that PAPSS2 knockdown did not sensitize NSCLC cells to CDDP treatment and its expression was unaffected at the protein level when PAPSS1 expressed was silenced (Figure 3.2B). Although increasing information about the importance of PAPSS has become available in recent years, the role of sulfonation in cancer and other human diseases is poorly understood, particularly in the context of sulfonation within the nucleus. Bruce et al. have uncovered PAPSS1 as a novel target for HIV infections and have speculated that sulfonation may play a role in epigenetic modifications of DNA in the nucleus [218]. In the context of cancer, we were the first to disclose that PAPSS1 silencing sensitizes NSCLC cells to multiple DNA damaging agents including platinum and non-platinum-based DNA cross linkers, topoisomerase I inhibitors, and radiation (Figure 3.8). The combination of PAPSS1 inhibition with these treatment agents results in greater accumulation of DNA damage that ultimately lead to cell death, suggesting that sulfonation might be involved directly or indirectly with the recognition of DNA damage or DNA damage repair [97]. To advance this work and to better justify studies focused on PAPSS1 as a therapeutic target, it was necessary to demonstrate that sensitization to CDDP is also observed in different model systems. The present study focused on a single cell line, A549, which were used in the original genome-wide screen that led to the identification of PAPSS1 as a potential therapeutic target. However, we grew these cells under a number of different experimental conditions to better understand how PAPSS1 knockdown alone impacted expansion of A549 cells in the presence and absence of low-dose CDDP.
For example, in an *in vivo* setting, tumours often outgrow the ability of existing and newly formed blood vessels to provide oxygen and nutrients [219]. This also means that following treatment with chemotherapeutics, established tumours are exposed to drug concentration gradients (high near the blood vessels and low as the distance from the blood vessel increases) [213]. Thus, it is known that tumour cells that are distant from blood vessels are exposed to sub-lethal/ ineffective concentrations of chemotherapeutics, which this contributes to treatment failures. Therefore, it is critically important to understand whether PAPSS1 engendered sensitization to DNA-damaging agents is observed when cells are grown under hypoxic and/or low nutrient conditions as well as in three dimensional models (e.g. spheroids, zebrafish xenografts and tumours grown in mice) where the structure of the growing tumours limits drug penetration.

In one aspect of these studies, siRNA-transfected A549 cells were conditioned to serum-starvation and/or chronic hypoxia (1.0% oxygen) and then treated with a range of CDDP concentrations while being maintained under these stress conditions. The results obtained under normal conditions were consistent with our previous report showing a 5-fold reduction in the CDDP IC$_{50}$ in PAPSS1-silenced cells that have been treated for 72 hours (see Figure 4.1). Importantly, when PAPSS1-silenced cells were serum-starved, oxygen-deprived, or starved and oxygen-deprived they were even more sensitive to CDDP treatment compared to the same cells that were cultured under normoxic conditions in the presence of 10% serum. The magnitude of the effect increased from 5-fold under well-fed, normoxic conditions to 50-fold under serum-starved, hypoxic conditions.

In another aspect of these studies multi-cellular spheroids were grown of A549 cells with and without depleted levels of PAPSS1. Spheroids have been used to model the three-dimensional structures which form *in vivo*, consisting only of tumour cells with no host cells supporting tumour progression [220, 221]. Spheroids consist of an outer layer of actively proliferating cells, an intermediate zone of hypoxic, viable cells, and an inner necrotic core [220, 222]. Their growth characteristics and sensitivity to anticancer agents are believed to be much more representative of tumours *in vivo* when compared to tumour cells grown in monolayer cultures [223, 224]. In these spheroid studies, the A549 cells used expressed a red-fluorescent protein so that cell proliferation could be tracked as the spheroids grew. When evaluating formation of spheroids grown from PAPSS1-silenced
cells over 60 hours, reduced fluorescence intensity was observed as compared to controls, suggesting decreased proliferation or increased cell death (see Figure 4.2). This was consistent with our previous studies which showed that PAPSS1 knockdown alone affects long-term cell viability when using colony formation assays [97]; an effect that is not reflected in short-term cytotoxicity assays performed with monolayer cultures. Although PAPSS1-silenced cells were able to consistently form spheroids, it is possible that the spheroids formed are not exactly the same as those formed from the scramble controls. However, at the time of CDDP addition, the spheroids formed from the PAPSS1-silenced cells and scramble cells were comparable in size, approximately 350-400 µm in diameter. Depletion of PAPSS1 sensitized cells to low-dose CDDP in the spheroid model, while high dose CDDP continued to be effective against both PAPSS1-silenced and scramble spheroids (Figure 4.2). These results are consistent with our belief that we have uncovered a “synthetic sick” interaction where PAPSS1 inhibition impairs the fitness of the cancer cells which leads to increased sensitivity to doses of CDDP that would not normally provide therapeutic benefits [15].

To further validate these results, both zebrafish and murine xenograft models derived from A549 cells with normal and depleted PAPSS1 were completed. Zebrafish models have become a powerful tool for rapid and cost-effective validation of anticancer therapies [225] and the zebrafish xenograft model described here was previously used to graft human leukemia and sarcoma cell lines into embryos [216, 226, 227]. Following the inoculation of siRNA-transfected cells, the PAPSS1-silenced and control cells, it was shown that all cells proliferated at similar rates in the zebrafish embryos (see Figure 4.3). The xenografts derived from PAPSS1-deficient cells were more sensitive to low doses of CDDP (0.25 mM and 0.125 mM) when compared to the controls. Note that the tolerated dose of CDDP in zebrafish is much higher than that used in tissue culture and treatment was added by simply diluting CDDP in the egg water in which the fish swims. The total amount of CDDP taken up by cancer cells within zebrafish was not assessed. Overall, the results presented using this model are somewhat limited as growth was compared at essentially one time point, equivalent to when the controls doubled in size. Therefore we also determined whether PAPSS-1 silencing influenced treatment responses in models grown in mice. For the purpose of generating murine xenografts, a cell line that stably expresses shRNA which
targets PAPSS1 was created (see Methods 4.2.11). In culture, under selection conditions, these cells did not regain gene expression over multiple passages. In contrast, when established tumours were harvested from animals inoculated with cells modified with PAPSS1-targeting shRNA, the expression of PAPSS1 was comparable to controls (see Figure 4.4). This compromised the design of the experiment and forced us to evaluate the effect of CDDP treatment at a time when PAPSS1 suppression was confirmed (Day 7). The results were consistent with the rest of the data presented in this manuscript and clearly indicate that the tumours derived from cells with depleted PAPSS1 are more sensitive (compared to controls) when treated with CDDP (see Figure 4.5).

At this stage, we believe that the results presented here established in multiple model systems which were generated using A549 cells that PAPSS1 depletion increases sensitivity to CDDP. Previously, we have demonstrated that enhanced sensitivity is seen in multiple NSCLC cell lines (see Figure 3.4) and these has been extended to multiple ovarian cancer and colorectal cell lines including ones that exhibit decreased sensitivity to platinums (unpublished observations). Further, we have shown that the chemosensitization effects seen in cancer cell lines with depleted PAPSS1 are observed for multiple DNA damaging agents as well as radiation (see Figure 3.8). What is not clear at this stage are the exact mechanisms responsible for enhanced sensitivity, but we have argued that this must be associated with changes in sulfonated proteins within the nucleus and some of these proteins are important to DNA repair signalling pathways. Further studies are also being initiated to establish whether chemosensitization is due to loss of one or both of the enzymatic functions of PAPSS1. A small molecule inhibitor screen will be completed to identify small molecular weight inhibitors of the ATP sulfurylase and kinase functions of this protein. Alternatively, the effects may not be due to the loss of PAPSS1 enzymatic activity but instead are a result of the loss of specific protein-protein interactions. To address this, we hope to use tandem affinity purification assays to identify PAPSS1-binding partners [228]. Such interactions, if confirmed to be causal of chemosensitization, could then be targeted through the development of peptide therapeutics that block the protein-protein interactions [229]. We believe that a small molecule or peptide targeting PAPSS1 will have broad applications in a wide range of cancers that are currently treated with CDDP and other DNA damaging agents as part of standard of care.
5. Summarizing Discussion and Future Directions

5.1 Summary of Results

In Chapter 2, the whole genome siRNA screen and the Affymetrix microarray study were presented. When the two datasets were overlaid, a list of 9 genes, upregulated following exposure to low-dose cisplatin (CDDP), were found to rank within the top 10% of the siRNA screen. Five of these genes were selected based on their potential contribution to cancer development and treatment response according to the literature and validation studies with these 5 genes were completed where enhanced CDDP activity was measured using colony formation assays. The validation studies performed in two NSCLC cell lines revealed CABYR and RRM2B as candidate gene targets that may be suppressed to enhance the activity of low-dose CDDP. These results support the original hypothesis of this thesis: blocking certain changes in gene expression induced by exposure to low-dose CDDP can augment the cytotoxic effects of CDDP. Although these two targets appear to be promising, they are currently not druggable. The pharmaceutical development of gene targets that arise from the siRNA screen, which is beyond the scope of this thesis, may nonetheless be delayed due to several challenges which should be considered. These challenges are described in detail in section 5.2 below.

Chapter 3 and 4 of the thesis focused on a target that arose from analyzing a kinase subset of the siRNA screen. Similar to the targets identified in Chapter 2, no small molecule inhibitor exists for PAPSS1. Although PAPSS1 was not differentially expressed (microarray study) when cells were initially exposed to CDDP, it is the most promising cancer-specific target identified through this research. PAPSS1 depletion engendered the greatest level of CDDP potentiation amongst all validated targets. Furthermore, PAPSS1 depletion engendered the greatest level of CDDP potentiation amongst all validated targets. Furthermore, PAPSS1 suppression enhanced the activity of multiple DNA damaging agents including radiation (see Chapter 3). When PAPSS1 is depleted and cells are exposed to DNA damaging agents, there is an accumulation of double-stranded DNA breaks as supported by the presence of numerous γH2AX foci in the nuclei of treated cells (see Figure 3.7). To determine whether PAPSS1 is worth pursuing as a therapeutic target, its effect on CDDP activity was further explored in NSCLC cells grown as 3D spheroids and in zebrafish, and mouse tumour models (see Chapter 4). Our results demonstrated that PAPSS-1 depletion significantly enhanced the
activity of low-dose CDDP when A549 cells were grown as spheroids or as xenografts grown in zebrafish. Furthermore, cells expressing PAPSS1-targeting shRNAs and inoculated in immune-compromised mice responded better than controls when those animals were treated with an effective, but non-curative, dose of CDDP. Note that a caveat in this target validation process is that rescue experiments as a confirmation of the on-target effects of PAPSS1 inhibition were not presented due to three reasons: 1) The shRNA-modified cell line could only achieved ~50% reduction in PAPSS1 level, making it unreliable for the knock-in experiment. 2) When a double-transfection (siRNA transfection followed by transfection of a PAPSS1-overexpressing vector) was attempted, the transfection efficiency of the plasmid (~1%) made it impossible to perform transient rescue experiments. 3) The product of the PAPSS reaction, PAPS cannot be supplemented to rescue the effects of the knockdown as the chemical compound is unstable and degrades within 30 minutes at 37°C.

When the research described in Chapter 3 was reviewed for publication, one of the external reviewers asked whether PAPSS1 depletion would sensitize NSCLC cells to CDDP under conditions where the cells were stressed under nutrient depletion or hypoxia. It is clear that the tumour microenvironment plays an important role in nutrient and oxygen availability to tumour cells, and it is in these regions within the tumor that may be exposed to lower doses of CDDP. The validation studies were therefore extended to test this idea and the results demonstrated that PAPSS1 knockdown significantly augmented the activity of CDDP when A549 cells are stressed by serum starvation and hypoxia. While all of these validation studies support the hypothesis that PAPSS1 inhibition enhances the activity of CDDP in NSCLC, the exact mechanism of action is yet to be defined. This presents a significant challenge in translating this target for clinical applications. In hopes of understanding how PAPSS1 plays a role in cancer biology, we first explored the role of PAPSS1 in normal cell biology in the literature. This led to the recognition that the significance of sulfonation and its roles in human diseases have, in general, been under-appreciated. Section 5.3 provides a review on the current understanding of sulfonation reactions and their roles in human diseases, with an emphasis on cancer. It is evident that there is a lack of knowledge with regards to sulfonation reactions, particularly those mediated by PAPSS1 in the nucleus of cells and this will likely be a very productive avenue of research in the future.
5.2 Translating Synthetic lethality for Clinical Applications

Although numerous synthetic lethal interactions have been discovered through genetic and chemical screening approaches, many have yet to achieve clinical success. Translating synthetic lethal/sick discoveries to the clinic can be challenging depending on various factors such as the availability of small molecule inhibitors and whether the novel drug combination is more efficacious than existing standard treatments. We have identified several factors that should be considered when translating synthetic lethal interactions to therapeutic strategies as well as pharmacological considerations that are important for developing synthetic lethality-based drug combinations for cancer patients. These factors are summarized in Figures 5.1 and 5.2.

![Figure 5.1. Considerations when validating synthetic lethal targets.](image)

Several factors should be considered when deciding whether or not to translate a synthetic lethal discovery to therapeutics. If the target was discovered from an RNAi screen, off-target effects should be eliminated by testing individual siRNA duplexes, using pools of siRNAs, or even testing the interaction using small molecules if available (A). Secondly, the synthetic lethality should be verified in a panel of cell lines for the indication(s) of interest to assess potential applications of the therapeutic strategy of interest (B). The therapeutic window should also be assessed to ensure that synthetic lethality occurs in a cancer-specific manner (C). When developing pharmaceuticals for the target of interest, it is crucial to understand whether it is the enzymatic activity or a specific interaction that is responsible for the synthetic lethality observed (D). Finally, synthetic lethality might be dependent
on the extent of genetic alteration. This dose dependency should be explored and addressed when designing and developing therapeutics for synthetic lethal targets (E).

**Figure 5.2. In Vivo Considerations for Synthetic Lethal Therapeutics.** When using two or more therapeutics, it is important to determine the drug combination ratios at which synergy occur (A). This should be done in a panel of cell lines for the indication(s) of interest. Synergism may also be dependent on the timing of the administration of the different therapeutics (B). Another challenge that needs to be addressed is the issue associated with drug penetration into the entire tumour (C). As a result of poorly organized vasculature, concentration gradients will be generated upon treatment and outcomes of synthetic lethal approaches may be limited by the inability to induce sufficient genetic alterations in all cells of the targeted population. Finally, while synthetic lethal approaches are promising, certain populations of the tumour may survive treatment due to intra-tumoural heterogeneity which makes them insensitive to the specific treatment regimen (D).

### 5.2.1 Considerations for Translating Synthetic Lethal Interactions to Therapeutic Strategies

When validating hits from RNAi screens, it is important to ensure that the phenotypic observations from gene-knockdown are not due to off-target effects (Figure 5.1A). While mRNA sequences that perfectly match the siRNA guide strand are cleaved by the RNAi machinery, off-target silencing could also occur where the mRNA has slight mismatches with the siRNA template, particularly in the 3’-UTR, giving rise to false positives due to unintended microRNA-like activities. These off-target effects could be minimized through
strategies such as chemical modification of the siRNA duplexes and utilization of pooled sequences [230]. Validation of the on-target effects is also necessary through the use of multiple RNAi sequences to eliminate sequence-specific effects and “rescue” experiments using cDNAs lacking siRNA-binding sites to exogenously express the putative target in the presence of endogenous gene knockdown with RNAi. Once the on-target effects are confirmed, it is crucial to test for synthetic lethality in a range of cell lines and relevant disease models to consider and assess the potential of targeting the interaction of interest in different subtypes of tumours from a specific organ or even in a wide range of solid and hematologic tumours (Figure 5.1B). The next step is to assess the therapeutic window associated with the synthetic lethal interaction. In cases where the synthetic lethal interaction is specific to an oncogenic or loss-of-function genetic background, it would be ideal that treatment causes little or no effect on cells expressing the wild-type version of the gene. The BRCA and PARP interaction is an example where PARP inhibitors are particularly effective against BRCA2-deficient tumours as demonstrated by Bryant et al [47]. Tumours harbouring wild-type BRCA2 or BRCA2-deficient tumours with BRCA2 overexpression did not respond to the treatment.

The widening of the synthetic lethality window (Figure 5.1C) should be carefully examined when validating chemo-sensitizing targets where genetic deficiency is introduced globally to enhance the activity of another drug. Firstly, gene knockdown alone should not be deleterious to the viability of normal cells. Secondly, chemosensitization should be selective for cancer cells. As an example, the target described in Chapters 3 and 4, PAPSS1, was found to enhance the activity of various DNA damaging agents in NSCLC cells [62]. We demonstrated in this study that knockdown of the gene did not sensitize normal bronchial epithelial cells to CDDP treatment while a five-fold reduction in the CDDP IC₅₀ was achieved in NSCLC cells (see Figures 3.4 and 3.2, respectively).

When developing small molecules based on the findings from an RNAi screen, it is important to understand the specific function of the gene product (Figure 5.1D) involved in the synthetic lethal interaction. Since transcription factors and other non-enzyme targets are generally considered “undruggable”, candidates with enzymatic activities such as kinases are prioritized for target development with the assumption that the kinase activity is responsible for the synthetic lethality observed in the RNAi screen. In
2009, Scholl et al. discovered from an RNAi screen that KRAS-driven cancers are dependent on a gene that encodes for a serine/threonine protein kinase STK33 [65]. In 2012, Luo et al. developed a potent and selective kinase inhibitor for STK33 which failed to reproduce the synthetic lethality observed in the original screen [231]. This could be due to the fact that STK33 has other non-kinase functions that are critical to the viability of KRAS-driven cancer cells, for example protein scaffolding. This could be explored through co-immunoprecipitation or with more advanced approaches such as tandem affinity purification during target validation as means to identify protein-protein interactions [232]. While small molecule inhibitors may be developed to inhibit the enzymatic activity of a candidate target, peptide therapeutics could be used to inhibit specific protein-protein interactions important for synthetic lethality [233]. This rather novel therapeutic area also opens up opportunities for targeting the traditionally “undruggable” hits from synthetic lethal screens.

Finally, targets discovered in yeast studies are based on complete gene knockouts; however, RNAi screens utilizing siRNA or shRNA approaches rarely eliminate the gene product completely. It is therefore important to determine the minimal level of protein inhibition necessary to achieve the desired synthetic lethal/sick effect. For instance, our studies on PAPSS1 showed that sensitization to CDDP treatment occurred in a siRNA dose-dependent manner (Figure 3.3). At the protein level, at least 80% inhibition relative to scramble controls was necessary to achieve a meaningful improvement in CDDP activity. While the extent to which the protein activity/interaction is inhibited can be determined in vitro and adjusted using medicinal chemistry, this dose dependency (Figure 5.1E) will be challenged when tested in vivo, as discussed in the following section.

### 5.2.2 Optimizing Multidrug Combinations to Induce Synthetic Lethality

As mentioned earlier, the success of multidrug combinations is largely dependent on the combinatorial effect being synergistic or synthetic sick in the context of synthetic lethality. Traditionally, drug combinations were given at maximally tolerated doses to achieve the greatest therapeutic effects. However, studies in the last decade have found that drug combinations display drug-ratio-dependent synergy (Figure 5.2A) [234]. For instance,
the combination of CDDP and irinotecan, which is an approved combination for the treatment of lung cancer, was screened by Tardi et al. in a panel of 20 cell lines over a range of drug ratio [235]. Their study indicated that an antagonistic region (irinotecan/CDDP molar ratios 1:2 to 4:1) was consistently detected in these cell lines. The regions of synergy (<1:2 and >4:1) were conserved in vivo. These results raise an important consideration that drug combinations should be given at the optimal synergistic ratios. However, different drugs exhibit different ADME (absorption, distribution, metabolism, and excretion) profiles and maintenance of synergistic drug ratios in vivo is necessary to achieve the desired synergistic effects. This problem can be addressed through the use of drug delivery technology. By co-encapsulating the two drugs at the optimally synergistic ratio into nanocarriers such as liposomes, the pharmacokinetic profile is controlled to maintain the drug ratio in vivo [234]. As an example, VYXEOS (CPX-351) is a liposomal formulation comprising cytarabine and daunorubicin (5:1 molar ratio) that was developed using the Combiplex® technology for the treatment of acute myeloid leukemia (AML)[236-238]. VYXEOS is currently in Phase III clinical trial as a potential replacement for the standard of care 7+3 cytarabine/daunorubicin treatment [239].

While drug combination ratios must be optimized when the drugs are given concurrently, some drug interactions benefit from sequential treatment. This schedule-dependent synergy (Figure 5.2B) should also be considered when designing and developing drug combinations. For instance, in a study conducted by Li et al., the use of pemetrexed followed by erlotinib or the use of these two drugs concurrently resulted in synergism in NSCLC cells [150]. However, the combination is antagonistic when erlotinib is given before pemetrexed. In another previous study, the use of the liposomal irinotecan formulation Irinophore C™ in combination with 5-FU concurrently resulted in high levels of toxicity which is eliminated when 5-FU is administered sequentially following Irinophore C™ in colorectal cancer models [240, 241]. Similarly, a phase III node-positive breast cancer trial demonstrated significantly better overall survival when patients were given doxorubicin and docetaxel sequentially relative to concurrent chemotherapy [242].

When translating synthetic lethality to therapeutic strategies, it is imperative to consider intra-tumoural heterogeneity as well as the tumour microenvironment. The tumour microenvironment has long been known to have significant impact on drug penetration
(Figure 5.2C) leading to treatment failures [243]. Due to the poorly organized vasculature in tumours, drug treatment with small molecules create concentration gradients that lead to reduced drug exposure at certain regions of the tumour. While screening strategies have been used to identify synthetic lethal gene partners that can be inhibited to enhance the cytotoxic effects of low-dose chemotherapeutics, these therapeutic strategies are limited by the same drug penetration issue. As mentioned earlier, a minimal level of gene knockdown would be required to have sufficient inhibition at the protein level to achieve the desired synthetic lethal or synthetic sick effect, as demonstrated by our PAPSS1 example. One challenge associated with the future development of gene, peptide, or small molecule therapy against this target would be our ability to achieve 80% PAPSS1 knockdown in all regions of tumours that are exposed to low-dose CDDP due to limitations in drug penetration. Although there will always be a drug concentration gradient, the effects could potentially be mitigated through the use of drug delivery systems. As an example, liposomal formulations of doxorubicin, which are clinically approved, have shown to be efficacious through increased circulation lifetime leading to increased drug accumulation at the tumour site [244, 245]. Although concentration gradients would still be generated in that case, a greater amount of drug would be available even at hypoxic or nutrient-deprived regions due to greater overall exposure to the therapeutic agent.

In terms of future perspectives, synthetic lethality is a promising approach at the cellular level and even at the population level given that the tumour population is clonal. In reality, however, intra-tumoural heterogeneity (Figure 5.2D) contributes tremendously to the challenge of curing lung cancer. By use of chemotherapy and/or targeted agents, one treatment regimen could potentially eradicate a large population of lung tumour cells which also removes the selective pressure against existing tumour cells that do not proliferate as rapidly but are resistant to the given treatment. Even in the context of tumours with driver mutations, which could potentially be targeted using synthetic lethal strategies, not all tumour cells would harbour the specific driver mutation simply due to the countless mutations acquired through numerous generations. Nonetheless, synthetic lethal strategies, if applied early, are extremely promising in extending patient survival. With the use of other advanced technologies, such as post-treatment sequencing of tumour biopsies, continuous applications of synthetic lethal strategies will allow the disease to be
managed for a longer period of time, ultimately making lung cancer a chronic, instead of a terminal, illness.

5.3 Sulfonation, an Underexploited Area: From Skeletal Development to Infectious Diseases and Cancer

Sulfonation is one of the most abundant cellular reactions modifying a wide range of xenobiotics as well as endogenous molecules which regulate important biological processes including blood clotting, formation of connective tissues, and functionality of secreted proteins, hormones, and signaling molecules. Sulfonation is ubiquitous in all tissues and widespread in nature (plants, animals, and microorganisms). Although sulfoconjugates were discovered over a century ago when in 1875 Baumann isolated phenyl sulfate in the urine of a patient given phenol as an antiseptic, the significance of sulfonation and its roles in human diseases have been underappreciated until recent years[198]. Here, we provide a current overview of the significance of sulfonation reactions in a variety of biological functions, medical conditions (with emphasis on cancer) and we discuss research areas that warrant further attention if we are to fully understand how deficiencies in sulfonation could impact human health which, in turn, could help define treatments to effect improvements in health.

5.3.1 Evolution of Sulfonation

Sulfonation plays an essential role in the biotransformation of hormones, neurotransmitters, and endogenous compounds and xenobiotics. Sulfonation reactions, catalyzed by sulfotransferases, involve the transfer of a sulfonate group (SO$_3^-$) from the obligate sulfonate donor 3’-phosphoadenosine-5’-phosphosulfate (PAPS) to a hydroxyl or an amino group [191, 246, 247]. In humans, PAPS is the biologically active form of sulfate; biosynthesized by the enzyme 3’-phosphoadenosine-5’-phosphosulfate synthase (PAPSS) [191]. The synthesis of PAPS involves two reactions: inorganic sulfate is first converted to adenosine-5’-phosphosulfate (APS) by ATP sulfurylase and then the intermediate molecule is phosphorylated by the APS kinase to form PAPS [198, 248] (Figure 5.3). In prokaryotes, fungi, and plants, synthesis of PAPS is performed by two separate enzymes [249-253]. In animals, however, the ATP sulfurylase and the APS kinase are encoded by the same gene
and translated into a single polypeptide which forms the dual-function enzyme PAPSS [179, 248]. Both APS and PAPS are activated sulfuryl donors that possess a phospho-sulfate anhydride bond [179]. Phototrophic bacteria, algae, and some plants are known to utilize APS for the synthesis of the sulfur-containing amino acids cysteine and methionine via the assimilatory sulfate reduction pathway while chemotrophic bacteria, fungi, and some higher plants use PAPS [179, 254, 255]. The specificity for APS or PAPS is dependent on the presence of an iron-sulfur cluster in the sulfate-reducing enzymes of the organism [255]. Interestingly, these sulfate reduction pathways are not present in humans and other animals, making methionine an essential amino acid that can only be obtained through dietary sources [179, 255]. Instead of amino acid synthesis, PAPS in animals is used for a variety of sulfonation reactions (summarized in Figure 5.4) including the biotransformation of endo- and xeno-biotics, as described below. Hence, human PAPSS has become structurally and functionally specialized through evolution.

**Figure 5.3. Bioactivation of inorganic sulfate.** Inorganic sulfate is converted to the biologically active form PAPS (3'-phosphoadenosine 5'-phosphosulfate) by the dual-function enzyme PAPSS. Inorganic sulfate is first converted to adenosine 5'-phosphosulfate (APS) by ATP sulfurylase. The intermediate molecule APS is subsequently phosphorylated via the APS kinase domain of PAPSS to form PAPS. The structures were drawn using ChemSketch and are color-coded as follows: red = oxygen, cyan = carbon, blue = nitrogen, yellow = sulfur, grey = phosphorous)
Figure 5.4. Sulfonation reactions in human cells. In the nucleus, conversion of inorganic sulfate to PAPS is catalyzed by PAPSS1. In the cytoplasm, the same reaction is catalyzed by PAPSS2. The PAPS produced in the cytoplasm is used by cytosolic sulfotransferases to biotransform endo- and xeno-biotics. Cytosolic PAPS can also be transported to the golgi apparatus via the PAPS translocase, where tyrosine sulfonation of proteins and sulfo-conjugation of polysaccharides occur. Sulfonated molecules such as heparan sulfates (HS) may be secreted to the extracellular matrix or attached to cell surface proteins. Some of the HS may sequester growth factors that are released upon cleavage by heparanases and sulfatases.

As mentioned earlier, two PAPSS isoforms exist in humans: PAPSS1 and PAPSS2 [256]. While both isoforms are expressed ubiquitously, they differ in cellular localization and tissue distribution. PAPSS1 is localized to the nucleus while PAPSS2 is found primarily in the cytoplasm [192, 257]. PAPSS1 is required for the re-localization of PAPSS2 from the cytoplasm to the nucleus for additional PAPS production [257]. In terms of tissue distribution, PAPSS1 is the predominantly expressed isoform in brain and skin while PAPSS2 is most expressed in the liver, cartilage, and adrenal glands [179]. Relative distribution of the two isoforms varies in other tissues [179]. Deficiencies in the two
isoforms are associated with different medical conditions as discussed below [217]. Altogether, these findings support that while both PAPSS1 and PAPSS2 catalyze the production of the obligate sulfonate donor PAPS, the two isoforms have non-redundant functions.

5.3.2 **Sulfotransferases and Xenobiotic Metabolism**

Sulfonation is most commonly known to be associated with metabolism of xenobiotics which inactivates drugs such as acetaminophen by increasing their water solubility for excretion and decreasing their membrane permeability as well as biological activity through the addition of a charged moiety [247]. This modification is also partially responsible for drug resistance to chemotherapy in cancer treatments [258]. While PAPSS1 and PAPSS2 are responsible for the bioactivation of sulfate, sulfo-conjugation reactions are catalyzed by enzymes known as sulfotransferases (SULTs) [191, 247]. Sulfotransferases are mainly divided into two groups: they are either cytosolic or membrane-bound [247, 259]. Cytosolic sulfotransferases constitute the superfamily of enzymes known as SULTs which are involved in the sulfonation of xenobiotics and small endogenous compounds such as neurotransmitters and hormones. The membrane-bound sulfotransferases are found in the Golgi apparatus and are responsible for post-translational sulfonation of endogenous macromolecules such as proteins, lipids, and glycosaminoglycans [259-261]. Currently, 13 SULT isoforms have been identified in humans [262]. These SULTs are grouped into four different families (SULT1, 2, 4, and 6) and they differ in their substrate specificity and tissue distribution [191, 262, 263]. SULTs are expressed at high levels during fetal development in humans [264]. In fact, some isoforms are only or primarily expressed during the prenatal period. The localization, expression levels, and the substrates (endogenous and foreign) of each SULT isoform characterized thus far are described in several comprehensive reviews [247, 264, 265]. A recent review by Coughtrie describes the function and organization of the different human SULT families [266]. SULT1 enzymes catalyze the sulfonation of catecholamines and many other compounds [266]. SULT1A1 and SULT1B1 are the primary enzymes involved in the metabolism of xenobiotics in humans, making up nearly 70% of hepatic sulfotransferases. SULT1A1 is considered the major SULT isoform in human tissues as it is highly expressed in the liver and the gastrointestinal tract, conjugating small phenolic compounds such as estrogens,
phytoestrogens, and minoxidil [267]. There are three isoforms within the SULT1A subfamily which differ in substrate specificity and thermostability albeit sharing >90% sequence identity [268]. In general, SULT1 enzymes catalyze the sulfonation of phenolic compounds such as estrogen while SULT2 enzymes are selective for steroids such as cholesterol and bile acids [247, 262, 269, 270]. The physiological functions of SULT4 and SULT6 are poorly understood [262, 269].

Sulfonation has been recognized as a high-affinity, low-capacity conjugation system that relies heavily on the availability of PAPS, which is dependent on its synthesis, use, and degradation [198, 271]. PAPS levels vary in different tissues and are believed to limit the sulfonation capacity of various cell types [271]. While each SULT isoform is known to have different tissue distribution and affinity for specific classes of substrates, it is also believed that PAPS concentrations are important in regulating substrate selectivity. Interesting studies by Cook et al. suggest that nucleotide binding triggers a gating mechanism that affects substrate selectivity [272]. Following nucleotide binding, there is a conformational change in the protein that limits substrate access to the catalytic domain. The authors showed in silico predictions of how saturating concentrations of PAPS could substantially decrease the affinity of SULT2A1 for large substrates. Whether these gating mechanisms apply to all human SULTs and how exactly substrate selectivity is regulated in each isoform remain to be investigated.

In most cases, sulfoconjugation is associated with detoxification. Some compounds, however, are bioactivated upon sulfonation by sulfotransferases [247]. Sulfonation could result in the generation of reactive electrophiles that can bind DNA, eliciting a mutagenic or even carcinogenic response [260, 263]. Based on screens conducted using recombinant bacteria, mammalian cell lines, as well as cell-free systems, about 100 compounds were identified to be genotoxic upon sulfonation by SULTs [263, 273]. Many studies have also shown that brachymorphic mice, which suffer from a PAPSS2 genetic defect (like patients with Pakistani spondyloepimetaphyseal dysplasia), are more resistant to tumorigenesis when exposed to procarcinogens known to be activated via sulfonation [274]. It is also possible that sulfo-concarinogens may enhance the therapeutic activity of certain drugs. Minoxidil, an anti-hypertensive and hair growth-stimulating drug, is one such example where the sulfate metabolite is responsible for its biological activity [260, 275, 276].
5.3.3 Tyrosine Sulfonation

Aside from biotransformation of xenobiotics, sulfonation is also the most abundant post-translational modification of tyrosine residues [277]. Overall, about 1% of the tyrosines are sulfonated [278]. While sulfonation is the appropriate term to describe the transfer of sulfonate groups (SO$_3^-$), the same reaction has been described widely as sulfation in the literature, particularly when discussing proteins and proteoglycans that have been sulfonated (ie. tyrosine-sulfated proteins and heparan sulfates). Here, we will use the term “sulfates” when referring to sulfonated tyrosine residues and glycosaminoglycans as these structures are commonly referred to as sulfates in the literature.

Currently, there is no evidence of protein tyrosine sulfonation (PTS) in yeasts and in prokaryotes, suggesting that PTS first appeared in multicellular eukaryotes [278, 279]. Tyrosylprotein sulfotransferase (TPST), the enzyme responsible for PTS, resides in the trans Golgi and PAPS, the obligate substrate for the reaction, is transported from the cytoplasm to the Golgi via a PAPS translocase [278, 280-282]. PTS has a multitude of biological functions, many of which are still being characterized [259]. PTS can be important for the biological activity of certain neuropeptides. For instance, sulfonated cholecsytoskinin, a hormone important for the secretion of digestive enzymes, is at least 200 times more active than its unsulfonated counterpart [278]. Translated proteins can also be modified by PTS to diversify their functionality. As an example, gastrin normally regulates the secretion of gastric acid. When sulfonated by PTS, gastrin can also function as a pancreatic secretagog [283]. The extent of proteolytic processing of gastrin is also associated with PTS, suggesting that PTS can regulate proteolytic cleavage [278].

With increasing interests in the biological roles of PTS, studies have started to focus on the importance of PTS in mediating the immune response [279, 284-286]. At sites of inflammation, adhesion of leukocytes to activated endothelium requires interactions between P-selectin on the endothelial cells and P-selectin glycoprotein ligand (PSGL)-1 on leukocytes. PTS is necessary at the tyrosine residues of PSGL-1 to facilitate this interaction [279, 284-286]. The presence of sulfonated tyrosine residues is also proving essential for proper blood clotting in response to vessel injuries as well as binding of chemokines to the chemokine receptors CCR5 and CXCR4 [279, 287].
5.3.4  **Heparan Sulfates**

Heparan sulfate (HS) is a polysaccharide that is produced by virtually all cells [288]. HS are often attached to proteins forming heparin sulfate proteoglycans (HSPGs) at the cell surface or in the extracellular matrix (ECM) [289]. Its basic structure consists of alternating hexuronic acid (D-glucuronic acid (GlcA) or L-iduronic acid (IdoA)) and D-glucosamine (GlcN) units [290]. The carbohydrate backbone is constructed as a polymer and then modified by a series of enzymes including glycosyltransferases, O-sulfotransferases, and an epimerase in the Golgi apparatus to form the final structure [288, 289]. HSPGs are known to be structurally diverse, with great variability in their chain lengths and sulfonation patterns. This gives rise to an immense number of HS species that can bind different proteins such as chemokines, growth factors, and enzymes and serve a variety of functions including immobilization, protection from proteolytic cleavage, as well as roles in embryonic development, angiogenesis, cell adhesion, blood coagulation, and lipid metabolism [289-292]. The biosynthesis and roles of HS can be further explored in depth in excellent previously published review articles [288, 289, 293-296].

5.3.5  **Sulfonation in the Context of Diseases and Cancer**

*Genetic Defects and Deregulation in the Sulfonation Pathway*

Genetic defects in the sulfonation pathway can have a wide range of effects. For instance, mutations in the diastrophic dysplasia sulfate transporter are associated with a lethal autosomal recessive disorder called achondrogenesis type 1B. This disorder is characterized by short limbs and pulmonary hypoplasia due to abnormal skeletal development [297-299]. Loss-of-function mutations in PAPSS2 are associated with a type of dwarfism called brachyolmia type 4, a non-lethal genetic disorder that affects the spine [300]. Depending on the specific causative mutations, PAPSS2 mutations can lead to brachyolmia or more severe skeletal disorders such as spondyloepimetaphyseal dysplasia Pakistani type, characterized by a number of abnormalities in the skeleton and the cartilage between long bones resulting in short stature and bowed legs [300].

In the context of tyrosine sulfation, there are two known human isoforms of TPST [301]. In mice, double knockouts of the two TPSTs result in post-natal pulmonary failure and death
as the lungs fail to expand [302]. Loss of TPST-2 activity causes hypothyroidism in mice, suggesting that the two isoforms have non-redundant substrate specificities and that tyrosine sulfation is necessary for normal pulmonary and thyroid gland functions [302, 303].

As mentioned above, PTS is important for inflammatory response. Specifically, in conditions associated with airway inflammation such as asthma and chronic obstructive pulmonary disease (COPD), PTS is prevalent [304]. It is known that binding of chemokines to chemokine receptors is essential in the regulation of leukocyte trafficking [305, 306]. Studies have demonstrated that the affinity of chemokine receptors to different chemokines is dependent on the sulfonation states of the tyrosine residues on the chemokine receptors [305]. In COPD patients, PSGL-1 is up-regulated on the surface of all leukocyte populations, where PTS plays a critical role in enhancing the interaction between immune cells and the bronchial endothelium [306].

**Roles of Sulfonation in Viral Infections**

The cellular sulfonation pathway is also known to be important for viral infections [218, 307]. HS is ubiquitous on the surfaces of cells and the highly sulfonated nature of HS provides ample negative charges that could interact with the positively charged viral proteins, promoting initial interactions between viruses and host cells[307]. In some cases, the interaction is much more specific. For instance, herpes simplex virus type 1 (HSV-1) binds to HS on target cells via envelope glycoproteins gB and gC, but viral entry is mediated through the interaction between viral glycoprotein D and a specific 3-O-sulfonated HS[307-310].

To date, HS are known to be involved in at least 16 different types of viral infections, including hepatitis C, human papillomavirus (HPV) and human immunodeficiency virus (HIV)[307]. In the case of HIV, the sulfonation pathway appears to be critical in multiple steps of viral infection. It has been established the binding of HIV involves an interaction between the envelope glycoprotein gp120 and syndecans, which are transmembrane HSPGs found on the cell surfaces of T-cells and macrophages [307, 311]. While HIV-1 only infects CD4+ cells, the attachment of the virus to the HS of a non-permissive cell actually aids the virus in retaining its infectivity for a longer period than it would otherwise as a
free virus [312]. This suggests that cells lacking CD4 expression may provide a reservoir for any bound HIV-1 [312].

Successful infection of HIV-1 virus requires expression of CD4 as well as the presence of specific co-receptors on the host cells. The chemokine receptor CCR5 is a major co-receptor that facilitates the entry of HIV-1 into target cells [287]. CXCR4 is another chemokine receptor that is commonly used by HIV-1 viruses as the infection progresses [313]. Based on studies completed by Farzan et al., both CCR5 and CXCR4 are sulfonated [287]. Specifically, when the tyrosine residues at the N terminus of CCR5 are mutated to phenylalanine and are not sulfonated, there is a marked decrease in the binding of CCR5 with MIP-1α, MIP-1β, and gp120/CD4 complexes, significantly reducing the ability of HIV-1 to enter their target cells [287]. It has also been recently established that PAPSS1 plays a critical role in gene expression from the long terminal repeat promoter following provirus establishment [218]. In this example, the transcriptional activity may be influenced by sulfonation in an epigenetic manner [218]. Although the mechanism is not fully understood, it is clear from the work of Bruce et al. that sulfonation in the nucleus is required for proper expression of LTR-driven genes which is needed for viral replication [218]. Finally, HIV-1-infected cells are known to release a protein called Tat (transactivator protein), the causative agent of AIDS (acquired immune deficiency syndrome), which is known to damage tissues and cells and is associated with neurotoxicity and increased risks of developing cancer [307, 314, 315]. Tat can also cause non-permissive cells to become susceptible to HIV infections [307]. The internalization of Tat into cells is facilitated, again, by HS [307, 315]. As summarized here using HIV as an example, sulfonation plays essential roles in viruses' ability to infect and complete their life cycles in humans. Also the structural diversity of HS is immense and extensive studies in this area are therefore necessary to fully understand how specific HS aids in the infections of different viruses.

**Sulfonation and Cancer**

While phosphorylation is extensively studied in cancer development and treatments, sulfonation has been largely overlooked in the context of oncology[316]. It is only in recent years that there is a growing body of evidence that individual differences in various genes of the sulfonation pathway may contribute to carcinogenesis and patient survival. For
instance, polymorphisms in SULT1E1, a sulfotransferase is involved in estrogen metabolism, are correlated with the survival of patients with estrogen-dependent cancers. Studies conducted by Hirata et al. and Rebbeck et al. demonstrated that polymorphisms in SULT1E1 are associated with greater endometrial cancer risks [317, 318]. In estrogen receptor (ER) positive breast cancers, tumorigenesis and disease progression rely on the presence of estrogen [319]. SULT1E1 is known to be overexpressed (relative to breast cancer cells) in normal human mammary epithelial cells [320]. In a recent study, Xu et al. demonstrated that overexpression of SULT1E1 and PAPSS1 can block estrogen-stimulated cell proliferation in MCF-7 breast cancer cells, while promoting apoptosis through upregulation of the pro-apoptotic gene bax [321]. It is important to note that the role of estrogen sulfonation in cancer patients could be complicated as some SULT isoforms have overlapping substrate specificity and need to be considered. For instance, SULT1A1 catalyzes the sulfonation of a variety of small phenolic compounds including estrogen and is known to be associated with breast cancer risks [322, 323]. Tamoxifen, an agent that is commonly used to treat breast cancer, is a prodrug that is metabolized by SULT1A1 to its activated metabolite 4-hydroxytamoxifen (4-OH TAM) in the liver. Polymorphism in SULT1A1 are known to exist and one particular variant SULT1A1*2 (where Arg at codon 213 is substituted with His), has been found to exist in some breast cancer patients. This enzyme has significantly lower enzymatic activity and thermostability [324]. While the investigators expected SULT1A1*2 to correlate with improved survival due to reduced sulfonation of 4-OH TAM they surprisingly found that the variant allele was associated with significantly poor survival in patients who were treated with tamoxifen [324]. This observation was supported by another study conducted by Wegman et al [325]. It was suggested that the sulfonated 4-OH TAM metabolite actually serves as a potent inducer of apoptosis, thus improving the survival of individuals with the SULT1A1 genotype that has higher enzymatic activity [326]. In another study, women bearing benign and malignant gynecological tumours were found to have a higher frequency of the common allele of SULT1A1, suggesting that there was an increase in endometrial cancer risk with greater SULT1A1 activity [327]. However, other studies have found either no or negative correlation between SULT1A1*2 and endometrial cancer risks. Clearly there is a need for further investigations [328-330].
Polymorphisms in SULT1A1 have also been studied in patients with estrogen-independent cancers. Several studies in lung cancer have demonstrated that the variant SULT1A1*2 allele is associated with increased risks of lung cancer, especially for smokers [331-333]. Interestingly, contrasting results were found in bladder cancer. Women and never smokers with the His (213) allele were found to have reduced risks of bladder cancer [334]. The variant allele even appears to provide some protective effects for smokers against bladder cancer [334, 335]. The same polymorphism is associated with significantly greater risks of upper urinary tract urothelial cell carcinoma, head and neck cancer, gastric cancer, and colorectal cancer, particularly in smokers and consumers of alcohol and red meat[336-339]. These findings suggest that SULT1A1 activity plays roles in carcinogenesis but in a tissue-specific manner.

Aside from SULTs, heparin sulfate proteoglycans and chondroitin sulfate proteoglycans (CSPGs) have also been shown to be associated with cancer. Chondroitin sulfates (CS) are sulfonated glycosaminoglycans complexed with core proteins to form CSPGs that reside at the cell surface and extracellular matrix[340]. CSs are synthesized during embryonic development as polysaccharides of alternating D-glucuronic acid and D-N-acetylgalactosamine units which are further modified via sulfonation [340, 341]. CSPGs are highly expressed in the vessels of brain tumours and the stroma of various types of cancer including melanoma, prostate, breast, testicular, colon, pancreatic, and gastric cancers and are known to play important roles in tumour growth and invasion [340-345]. The anionic nature of CS chains facilitates interactions between cells through binding of ligands and receptors that result in activation of signaling pathways that promote tumour growth and metastasis [340, 346, 347]. In head and neck cancer, patients appear to excrete chondroitin sulfates in their urine, which could be useful for diagnostic purposes [341]. Recently, Poh et al. have synthesized a library of CS disaccharides to evaluate the effects of different sulfonation patterns on breast cancer cell viability [348]. Their results suggest that the presence of specific sulfonation patterns could lead to growth inhibition in a triple negative breast cancer cell line. Asimakopoulou et al. have provided a review of some of the biological roles played by CS in different types of malignancies as well as an overview of several modified CSs that have been tested as targeting and therapeutic agents [340].
Similar to CSPGs, the abundance and diversity of HSPGs at normal and tumour cell surfaces and in the extracellular matrix affect cancer biology by initiating transformation of normal cells, modulating tumour growth, and promoting metastasis [349]. For instance, Glypican-3 (GPC3) is a HSPG that negatively regulates cell proliferation and survival [350]. Several studies have shown that GPC3 expression is reduced in mesothelioma, breast cancer, and ovarian cancer cells [349-352]. In other studies, induction of a sulfatase SULF2 was found in breast cancer and lung cancer [353, 354]. Sulf-2 expression is associated with activation of the Wnt signaling pathway which promotes cell proliferation [353, 355]. In contrast, suppression of Sulf-2 in cancer cells inhibited cell growth and even partially reversed transformation in vitro [353].

As mentioned earlier, different sulfonation patterns on HSPGs enable them to bind different molecules including growth factors. HSPGs therefore can sequester growth factors and release them upon degradation of their HS chains by heparanases or changes in sulfonation patterns by sulfatases, directly regulating cell proliferation[316, 349]. Vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGF) are growth factors that are also released from HSPGs by endosulfatases and heparanases in the extracellular matrix[316, 356]. Pericellular HSPGs can facilitate the binding of VEGF and FGF with their corresponding receptors on endothelial cells and activate these signaling pathways that are central to the process of angiogenesis [357]. While HSPGs can sequester or release pro-angiogenic factors, other HSPGs can inhibit angiogenesis by binding the anti-angiogenic factor endostatin. The ultimate stimulation or inhibition of angiogenesis is therefore a fine balance between the concentrations and binding affinities of pro- and anti-angiogenic factors, which are highly dependent on the HSPG profiles of cells in the tumour microenvironment [349, 357]. As an example, Cole et al. have demonstrated that down-regulation of HS 6-O-sulfotransferases 1 and 2 leads to reduced 6-O-sulfonation, reduced endothelial cell signaling and angiogenesis, and significantly delays the growth of ovarian cancer tumours in vivo [358]. A recent study conducted by Mao et al. reveals the role of the heparan sulfate sulfotransferase 3-OST3A (HS3ST3A) in regulating tumour growth in breast cancer by controlling the tumour microenvironment [359]. Depending on the tumour subtype, 3-OST3A expression could induce oncogenic or tumour-suppressive effects and even affect therapeutic responses by regulating the activity of FGFs. A similar
study was conducted by Kumar *et al.*, investigating the role of heparan sulfate 3-O-sulfotransferase 2 (HS3ST2) on the invasiveness of highly and low invasive breast cancer cells [360]. While FGFs are more commonly known to be involved in cancer cell proliferation, Jung *et al.* has recently shown that PAPSS2 depletion leads to undersulfonation of HSPGs, which in turn augments FGFR1 and Akt signaling, ultimately inducing premature cellular senescence, a tumour-suppressive mechanism that could lead to tumour clearance through an innate immune response [361].

In terms of tumour metastasis, HSPGs, again, play opposing roles. Along with collagen and laminin, HSPGs construct a protective barrier through tight cell-cell and cell-ECM adhesions [362]. Heparanases and other enzymes released by tumour cells help to modify these barriers as needed for invasion and metastasis [363-366]. Therefore, in most cases, tumour cells with low levels of HSPGs are correlated with high metastatic potential [362]. Specifically, studies in recent years have demonstrated that the expression of a specific HSPG, syndecan-1, is associated with changes in tumour cell morphology. Syndecan-1 is negatively correlated with metastatic potential in many different types of malignancies including mesothelioma, colon, lung, liver, breast, and head and neck cancer [362, 367-371]. The involvement of syndecan-1 in metastasis is explored in depth in a review written by Sanderson [362]. Since the interactions between tumour cells and the endothelium during metastasis are poorly understood, Martinez *et al.* conducted a study and demonstrated that cancer-associated glycosaminoglycans can serve as ligands for selectins in the endothelium and are recognized and bound based on sulfonation density and pH conditions [372]. These interactions help promote distant metastases.

While cell surface HSPGs are important for preventing tumour invasion, HSPGs in the ECM are involved in promoting metastasis. Extracellular HSPGs secreted by cells are known as perlecans while syndecans are cell surface HSPGs that could also be shed either into the ECM or into blood [362]. Studies have shown that suppression of perlecans decreases the invasiveness of metastatic melanoma and prostate cancer cells [373, 374]. While the mechanism of action of perlecans is yet to be defined, it has been suggested that perlecans and other HSPGs in the ECM may bind chemokines and growth factors, establishing gradients that direct the motility of migrating tumour cells [362].
As described above, HSPGs form a physical barrier with collagen to prevent invasion. Soluble HSPGs could also promote tumour cell invasion by interfering with cell-cell interactions. Studies suggest that exogenous HSs allowed non-invasive cells to become highly invasive in several rodent tumour and human leukemia cell lines [375, 376]. Once tumour cells have escaped from their primary site into the vasculature, their cell surface HSPGs may aid in their motility as well as their extravasation out of circulation [362]. For this reason, intravenous administrations of different species of HSs and anti-HSPG antibodies were tested in an attempt to block HS function in the vasculature and ultimately inhibit metastases [377-379].

In summary, tumour cells may secrete heparanases and other enzymes to degrade cell-surface HSPGs and ECM-bound HSPGs in the proximity to initiate the metastatic processes. Subsequently, cell surface HSPGs aid in migration, extravasation; promoting "seeding" at their metastatic sites. All of these steps involve a variety of HSPG species, enzymes, and signaling molecules that are dynamically regulated by the tumour cells. This suggests great opportunities for the development of therapeutics targeting HSPGs including the use of modified HSPGs, antibodies, and heparin. Heparin, an anticoagulant, is structurally similar to HSPGs and has been found to be anti-metastatic in multiple animal models for undefined reasons. These strategies are discussed in detail in several review articles [349, 379-382]. While the connection between HSPGs and cancer is well-established, there is still much to learn because the role is complex, depending on the tissue type, the presence of enzymes, growth factors, and other HSPGs, as well as the localization (cell-surface vs. ECM) and structural characteristics (sulfonation pattern) of each HSPG. It is also important to explore how the activity of PAPSS enzymes and the availability of PAPS, which are utilized in all sulfonation reactions, affects the structure and function of HSPGs.

5.3.6 Sulfonation in the Nucleus: The Uncharted Territory

Sulfonation is known to be involved in a broad spectrum of biological processes in both healthy and diseased cells. As indicated above, most studies have focused on the importance of sulfonation in cell-cell or cell-ECM communications and the modification of endo- and xenobiotics. However, very little is known about the sulfonation pathway in the nucleus, the control center of the cell. As mentioned above, two isoforms of PAPSS exist in
humans, both of which contain a nuclear localisation signal (NLS) for translocation from the cytoplasm to the nucleus of the cell [257]. The NLS is more efficient in PAPSS1 and subcellular localization studies conducted by Besset et al. have shown that PAPSS1 localizes to the nucleus while PAPSS2 is predominantly found in the cytoplasm [192]. When co-expressed with PAPSS1, PAPSS2 is relocated to the nucleus [192]. Therefore, PAPSS2 may relocate to the nucleus when a greater demand for PAPS needs to be met in the nucleus. These data suggest that there is a critically important role of the sulfonation pathway in the nucleus. The existence of a functional sulfonation pathway is further confirmed by studies completed by Bruce et al. where PAPSS1 was identified to be involved in suppressing transcriptional activities from the LTR promoter upon provirus establishment in the host cells [218].

In our laboratory, an siRNA screen led to PAPSS1 as a target that may potentiate non-small cell lung cancer (NSCLC) cells to CDDP treatment [97]. Validation studies further demonstrated that PAPSS1 can be suppressed to enhance the therapeutic activity of a range of DNA damaging agents, including radiation, other platinum-based agents, as well as topoisomerase I inhibitors [97]. The sensitization effects were observed in both platinum-sensitive and resistant cell lines via colony formation assays (Figure 5.5) [95, 97]. This sensitization was selective for cancer cells as strong inhibition of PAPSS1 at the protein level did not sensitize normal bronchial epithelial cells to cisplatin treatment [97]. Validation of PAPSS1 as a therapeutic target has been further demonstrated in multicellular tumour spheroid models as well as in zebrafish and rodent models (See Chapter 4). These results suggest that the sulfonation pathway plays an important role in the survival of cancer cells following exposure to chemotherapy.
Figure 5.5. PAPSS1 knockdown sensitizes ovarian cancer cells to platins. Cisplatin (CDDP) sensitive (A2780-S) and resistant (A2780-CP) A2780 and SKOV-3 cells were seeded in 6-well plates and transfected with a pool of three PAPSS1-targeting or non-targeting siRNA duplexes the following day. The empirically determined IC10 of the corresponding platinum agent based on a 72h cell viability assay was added to the cells 24 hours post-transfection (0.112, 1.24, 0.98 µM CDDP for A2780-S, -CP, and SKOV-3 respectively; 19.3 µM carboplatin (CBDCA) for SKOV-3). At 24 hours post-treatment, the cells were harvested and re-seeded for colony formation. The cells were subsequently incubated for 14 days undisturbed, after which the colonies were fixed and stained with 0.5% w/v crystal violet in 6.25% glutaraldehyde and counted. The plating efficiency (PE) was calculated using the equation (# colonies formed/#cells seeded)x100%. Representative images of the treatment conditions are shown in A and the PE values are plotted in B. All data are shown as mean ± SEM. Two-way ANOVA with Tukey adjustments for multiple tests comparison was used and the statistical significance of the sensitization effects of PAPSS1 knockdown is highlighted for each cell line (**** p<0.0001 for platin-treated scramble vs. PAPSS1-silenced cells).

According to the cBioPortal database, only approximately 7% of breast cancers and 2% of lung adenocarcinoma harbour PAPSS1 amplifications and mutations [97]. Currently, there is no database that can assess how PAPSS1 expression affects treatment response. A study conducted by Shih et al. has shown that single nucleotide polymorphisms in the ATP sulfurylase domain of PAPSS1 was correlated with poor survival in patients with familial or early onset hepatocellular carcinoma (HCC) [383]. The causal relationships between PAPSS1 and HCC survival has not been elucidated and the mechanism(s) by which PAPSS1 sensitizes NSCLC and ovarian cancer cells to DNA damage are yet to be defined. While our previous studies demonstrated increased accumulation of DNA damage when PAPSS1-deficient NSCLC cells were treated with CDDP and topotecan (see Figure 3.7), further research is needed to fully understand the mechanism of action as there is a lack of
knowledge of the roles of sulfonation in the nucleus of eukaryotic cells. Recent studies have shown that PAPSS1 is the more recently evolved isoform and that it can form a heterodimer with PAPSS2 [212, 384]. However, the function of this heterodimer and its potential role in regulating overall PAPS production is unclear. Therefore, although the PAPSS enzymes are starting to become recognized as potential contributors to cancer and other human diseases, research in the two enzymes that produce the substrate for all cellular sulfonation reactions is in its infancy and warrants further attention.

5.3.7 Future Perspectives on Sulfonation Research

Sulfonation is a key post-translational modification process that affects a tremendous number of biological activities through cell-cell and cell-matrix communication. There is a great amount of research concerning the roles of glycosaminoglycans and proteoglycans in diseases but it has been difficult to apply this knowledge to therapeutic applications because of the structural diversity and the tissue-specific nature of responses. There are growing interests in the roles of sulfotransferases in different types of malignancies; however, conflicting results suggest the need for greater sample sizes and more studies to understand how sulfotransferase activities affect cancer development, progression, and treatment responses in different organs. Protein tyrosine sulfonation is another area that is largely underexplored. Which proteins in the proteome are tyrosine-sulfonated? Part of this challenge concerns development of reliable methods for measuring sulfonated tyrosines on proteins. Some groups have been trying to predict sites of PTS by developing software and algorithms while more and more tyrosine sulfonated proteins are being identified in recent years [279, 385]. We are, however, still far from finding all tyrosine sulfonations and this, in turn, limits our ability to understand the role that tyrosine sulfonation plays in biology. Other questions that will need to be addressed in the future include: 1) How do cells alter HSPG expression on the cell surface? 2) How does the supply of PAPS affect the sulfonation efficiency of different sulfotransferases in different tissues? 3) Which processes require PAPS in the nucleus? From our perspective which is now focused on cancer, we believe that answering these questions will first require the recognition that sulfonated proteins and glycans in different cellular compartments and organs play important roles in cancer cell proliferation and survival.
Currently, a great amount of effort is focused on discovering inhibitors that target phosphorylated proteins to reduce signaling of hyper-activated pathways in cancer cells. There are, however, still many unexplored areas that could be of great importance in cancer treatment. Sulfonation is one where many correlations with tumorigenesis and patient survival have been recognized, but little is known about the mechanisms of action and casual relationships. The area is extremely broad and requires extensive research looking at the impact of sulfonation in various cellular compartments, tissue types, as well as its involvement in cancer cell mobility, invasion, and metastasis. Death from cancer is almost always attributed to metastasis [386] and there is a need to explore all potential strategies that may prevent or slow the development of metastasis. In this context, more research is needed to understand the role of PAPSS enzymes in cancer cell biology.

### 5.4 Conclusions and Future Directions

The results presented in this thesis support the use of synthetic lethal strategies and gene expression profiling to devise novel treatment combinations. The challenges of translating discoveries made through synthetic lethal screens are highlighted by the most interesting validated gene described in this thesis, PAPSS1. To better understand how PAPSS1 enhances the activity of DNA damaging agents, future studies must characterize the effects of PAPSS1 depletion on DNA damage response signaling. From a pharmaceutical development perspective, PAPSS1 activity assays could be developed and used to screen for small molecule inhibitors. Since it is unknown which sulfonation reactions are dependent on the production of PAPS specifically by PAPSS1, mass spectrometry could be used to determine which peptides are sulfonated in the presence and absence of PAPSS1 expression. Given that it is possible that the sensitization effects observed from PAPSS1 depletion could be due to loss of specific PAPSS1-protein interactions, affinity purification and mass spectrometry (AP-MS) methods need to be completed to identify interacting partners of PAPSS1. Ultimately, small molecule inhibitors and interaction-blocking peptides could be developed as therapeutics to be used in combination with various DNA damaging agents for the treatment of NSCLC as well as other cancers treated with DNA damaging agents.
References

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Appendix A: Supplemental Information

Supplemental Figures and Tables

Supplemental figures and tables are displayed in order of their citation in the thesis text.

Table A.1. Primers Used for RT-PCR

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>TaqMan Assay ID</th>
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<td>ALDH3A1</td>
<td>Hs00964880_m1</td>
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<tr>
<td>CDKN1A</td>
<td>Hs00355782_m1</td>
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<td>DUSP4</td>
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<td>FAS</td>
<td>Hs00236330_m1</td>
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<td>FDXR</td>
<td>Hs01031617_m1</td>
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<td>FNTA</td>
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<tr>
<td>GDF15</td>
<td>Hs00171132_m1</td>
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<td>Hs00968432_m1</td>
</tr>
<tr>
<td>TP53INP1</td>
<td>Hs00426835_g1</td>
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</tbody>
</table>

Figure A.1 CABYR Knockdown sensitizes A549 and H460 cells to various concentrations of cisplatin. Cells were seeded and transfected as described in the Methods section for colony formation assays. Different concentrations of cisplatin were added the following day for 24 hours. The cells were then harvested, trypsinized, and seeded at 500 cells/well and incubated for 14 days undisturbed. The colonies were then stained with crystal violet and counted. The plating efficiency was calculated using the formula PE = # colonies formed/#colonies plated. Data are averaged from all experimental trials and plotted as mean ± SEM. Statistical analyses were performed using two-way ANOVA followed by Sidak’s multiple comparisons test (*adjusted p-value <0.05). Our results show that CABYR knockdown alone has no effect on cell viability in A549 cells but enhances cisplatin activity significantly at low as well as high effect levels of cisplatin. In H460 cells, CABYR knockdown may have some effect of cell viability. The cisplatin enhancing effect is observed at low to mid-doses of cisplatin. At 0.5 μM, sensitization was observed but the difference was not statistically significant. At 1 and 2 μM, the drug alone exerts a lethal effect such that further sensitization was insignificant with CABYR silencing.
Table A.2 GO Enrichment Analysis of Over-Expressed Genes Based on Biological Process Ontology

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<th>Background Frequency</th>
<th>Sample frequency</th>
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<th>Fold Enrichment</th>
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<td>&gt; 5</td>
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<td>+</td>
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<td>negative regulation of fibroblast proliferation</td>
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<td>&gt; 5</td>
<td>+</td>
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<td>regulation of apoptotic process</td>
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<td>1.8</td>
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*The p-value is derived from the Pathway Studio software that determines the likelihood of the pathway being enriched from a random gene list of the same size.
Figure A.2. Differentially expressed genes are enriched in p53 signaling. Hierarchical clustering of the differentially expressed genes reveals pathway enrichment in p53 signaling, ascorbate and aldarate metabolism, and pentose and glucoronate interconversions.
Table A.3. siRNA screen results for the nucleotide excision repair pathway

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>% Viabilitya (Gene Knockdown + CDDP)</th>
<th>% Viability (Knockdown Only)</th>
<th>Gene Score Rankb</th>
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<tr>
<td>CCNH</td>
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<td>CDK7</td>
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<td>CUL4B</td>
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<td>XPC</td>
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aPercent viability is normalized to transfection controls as described for the siRNA screen analysis in the Methods section.
bGene score rank is the ranking based on the calculated gene score used to identify cisplatin-potentiating hits. The lower the value of the rank, the better the target is for enhancing cisplatin activity.
Genes highlighted in red are considered essential genes (knockdown = lethality) and genes highlighted in green are considered cisplatin-potentiators based on our analysis criteria.
### Table A.4. Top 20 kinases from siRNA screens

<table>
<thead>
<tr>
<th>Kinase Rank</th>
<th>PKS Kinase</th>
<th>WGS Kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PIP5K2A</td>
<td>PIP5K2A</td>
</tr>
<tr>
<td>2</td>
<td>CDC42BPA</td>
<td>STK16</td>
</tr>
<tr>
<td>3</td>
<td>PRKWNK4</td>
<td>SPEC2</td>
</tr>
<tr>
<td>4</td>
<td>LTK</td>
<td>PIK3R1</td>
</tr>
<tr>
<td>5</td>
<td>BLK</td>
<td>RPS6KA3</td>
</tr>
<tr>
<td>6</td>
<td>FN3K</td>
<td>PTK9L</td>
</tr>
<tr>
<td>7</td>
<td>PAPSS1</td>
<td>PRKAA2</td>
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<tr>
<td>8</td>
<td>MAP3K14</td>
<td>ALS2CR2</td>
</tr>
<tr>
<td>9</td>
<td>ALS2CR2</td>
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</tr>
<tr>
<td>10</td>
<td>FASTK</td>
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### Table A.5. Top 10 kinases from siRNA validation screens

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Figure A.3. Sensitization of A549 cells to cisplatin treatment is observed with three different PAPSS1-targeting siRNA. A549 cells transfected with 25 nM of either non-targeting (scramble; SCR) or PAPSS1-targeting siRNA were treated with low-dose cisplatin. The viable cell count at 72 hours following drug treatment is normalized such that 100% is equivalent to the cell viability of scramble-transfected, untreated controls. The three siRNA sequences used here are 1) 5’-GCAAATTCATGAAGTGCAAGTTTA-3’, 2) 5’-GATGCTGGCTTAGTGTGCATCACAA-3’, and 3) 5’-GGGAGTACTTGAGCAGTGCCTTCATTT-3’, targeting exons 4, 3, and 7 respectively. These three siRNA duplexes were pooled for the remaining validation studies to minimize off-target effects without compromising on-target knockdown. Statistical analyses were performed using one-way ANOVA followed by Tukey adjustment for multiple test comparisons (data plotted as mean ± SD; *p<0.05).

Table A.6. NSCLC cell line characteristics

<table>
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<th>Cell Line</th>
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Figure A.4. PAPSS1 knockdown causes variable loss in cell viability in different cell lines. Depending on the cell line, loss of PAPSS1 expression could result in cell death (data plotted as mean ± SEM; *p<0.05).

Figure A.5. Substantial knockdown could not be achieved in HLMVEC and no change in the cisplatin dose response was observed. Despite using the highest non-toxic dose of lipid-siRNA complex, the reduction of PAPSS1 mRNA levels was less than 70% (A) and changes in protein expression in the presence of the siRNA were minimal (B; 22.8% reduction in protein expression). Under these conditions, sensitivity to cisplatin did not differ between PAPSS1 and Scramble controls (C; data plotted as mean ± SEM). The sensitization observed compared to medium control could be attributed to lipid toxicity from the transfection.
Figure A.6. PAPSS1 silencing induces apoptosis and causes A549 cells to accumulate in the S phase in the presence of cisplatin. At 24 (A) and 48 (B) hours following cisplatin treatment, cells transfected with scramble siRNA arrest at the G2/M phase in a dose-dependent manner. Cells with reduced PAPSS1 expression are much more apoptotic relative to scramble controls and tend to accumulate at the G1/S phase. Data are plotted as mean ± SD from three replicates (*p<0.05 based on multiple t-tests comparing the means of scramble versus PAPSS1-depleted cells at each dose of CDDP).
Table A.7 Comparison of hits with previously published screens

<table>
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Table A.7. Results from thirty genes in the siRNA screen presented in our study are compared with that from four different screens conducted in human cells for cisplatin modulators by other groups. The lethality of gene knockdown is shown in two ways: numerical values and colour-coded circles: red = <50% viability; yellow = 51–74%; green = ≥ 75%
Figure A.7. Yeast lacking PAPS synthase activity is not sensitized to cisplatin. (A) Ten-fold spot dilution assays of the indicated strains grown on SD medium with or without cisplatin (250 μM) for 2 days at 30°C. The *met3Δmet14Δ* double mutant lacks both enzymatic components of yeast PAPSS, *elg1Δ* and *rad52Δ* serve as weak and strong cisplatin-sensitive controls respectively, and are compared to an isogenic wildtype (WT) strain. (B) The cisplatin cytotoxicity of *met3Δmet14Δ* double mutants was comparable to the WT control. The percentage viability of the cells after a pulse of high dose cisplatin was quantified, and normalized to untreated controls (mean ± SD; n=3).

Figure A.8. Pre-treatment with chlorate (50mM) causes ~2-fold leftward shift in the cisplatin dose response curve. A549 cells were pre-treated with medium or 50 mM sodium chlorate for 24 hours prior to cisplatin exposure for 72 hours. Data are plotted as mean ± SEM (n = 4). Individual doses were compared for statistical significance using the Student’s t-test (*p<0.05). Cells treated with sodium chlorate had a cisplatin IC₅₀ of about 1.2 μM, which is almost two-fold lower than that of the medium control (2.1 μM) (B; mean ± SEM; **p<0.01).
Supplemental Experimental Procedures

Yeast strains and methods

The haploid met3Δmet14Δ double mutant (MATa ura3Δ0 his3Δ1 met15Δ0 leu2Δ0 lys2Δ0 met3Δ::KanMX met14Δ::KanMX ) was generated in this study from the individual heterozygous diploid strains met3Δ::KanMX/MET3 and met14Δ::KanMX/MET14 [181]. Other haploid deletion strains were obtained from the MATa yeast knockout collection (Open Biosystems). Yeast strains were grown in YPD or synthetic dextrose (SD) medium containing only amino acids essential to complement the auxotrophies present in the strains.

Spot dilution assays [182, 184] and cisplatin cytotoxicity assays [183] were performed as described using the WT strain BY4741 as control. Cytotoxicity was assessed using a colony formation assay by plating cell dilutions on nonselective YPD agar plates following a 4 hour treatment of 6x10^6 cells with the indicated cisplatin concentration (0, 0.125, 0.25, 0.5, and 1.0 mM). The number of colonies was counted after 2 days of growth at 30°C.