Stony Brook University

A role for the chromatin remodeler *CHD3* in epithelial ovarian cancer platinum resistance

A dissertation presented

by

Noelle L. Cutter

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Doctor of Philosophy

in

Genetics

Stony Brook University

August 2011

Stony Brook University
The Graduate School

Noelle L. Cutter

We, the dissertation committee for the above candidate for the

Doctor of Philosophy degree, hereby recommend

Acceptance of this dissertation.

Robert Lucito-Dissertation Advisor
Assistant Professor, Cold Spring Harbor Laboratory

Linda VanAelst- Chairperson of Defense
Professor, Cold Spring Harbor Laboratory

Wei-Xing Zong
Assistant Professor, Stony Brook University

Stefan Tafrov
Biologist, Brookhaven National Laboratory

Nevenka Dimitrova
Research Fellow, Phillips
This dissertation is accepted by the Graduate School

Lawrence Martin
Dean of the Graduate School

Abstract of the Dissertation

A role for the chromatin remodeler *CHD3* in epithelial ovarian cancer platinum resistance

by

Noelle L. Cutter

Doctor of Philosophy

in

Genetics

Stony Brook University

2011

Epithelial ovarian cancer is the leading cause of death from gynecological malignancies. Currently platinum-based chemotherapy (such as cisplatin or carboplatin), coupled with a taxane based drug such as paclitaxel is the primary treatment for ovarian cancer. Approximately 25% of patients either present with or rapidly develop resistance to platinum based chemotherapy and all recurrent tumors are resistant. Epigenetic modifications have been associated with tumor formation and progression and may contribute to therapy response. We have screened a number of genes and family members for methylation difference between resistant patients and sensitive patients, and had shown that *CHD3* is silenced through an epigenetic mechanism in both ovarian cancer cell lines and primary ovarian tumors. Here we show that for *CHD3*, a member of the Mi-2 NuRD complex, that loss of expression causes increased resistance to the chemotherapy drugs Carboplatin and Cisplatin. Additionally, cell lines transcriptionally silenced for *CHD3* are more invasive, have migratory ability, and display a transformed epithelial to mesenchymal (EMT) phenotype. Cells display a striking decrease in growth, not caused

by apoptosis or senescence. Taken together, we provide the first evidence of a role for *CHD3* as an important epigenetic regulator of chemoresistance in ovarian cancer and hypothesize EMT as one of the underlying mechanisms. Furthermore, *CHD3* might represent a response predictor. Based on these findings we propose that *CHD3* will be a future therapeutic target for ovarian cancer.

Table of Contents

Chapter VII: Conclusions and Perspectives

List of Abbreviations

ABC	ATP binding cassette
ATCC	American type cell culture
ATP	Adenosine triphosphate
BLAST	Basic local alignment search tool
BrDU	Bromodeoxyuridine, 5-bromo-2'-deoxyuridine
CHD	Chromo-domain helicase binding protein
chIP	Chromatin immunopercipitation
CIC	Cancer initiating cells
CSC	Cancer stem cells
DAVID	Database for Annotation, Visualization and Integrated Discovery
DMSO	dimethylsulfoxide
DSB	Double strand break
EMT	Epithelial to mesenchymal transition
EOC	Epithelial ovarian cancer
GFP	Green fluorescent protein
GST	Glutathione *S*-transferase
HDAC	Histone deacetylase
HOSE	Human ovarian surface epithelial
i.p.	Intraperitoneal
LOH	Loss of heterozygosity
Me	Methylation
MMR	Mismatch repair

MOMA	Methylation oligonucleiotide microarray analysis
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
NER	Nucleotide excision repair
NuRD	Nucleosome remodeling domain
PARP	Poly (ADP-ribose) polymerase
PcG	Polycomb group
RISC	RNA-induced silencing complex
RNAi	RNA interference
RT-Q-PCR	Real time quantitative polymerase chain reaction
shRNA	Short hairpin RNA
STS	Staurosporin
TCGA	The cancer genome atlas
TSG	Tumor suppressor gene

List of Illustrations

List of Tables

Acknowledgements

I would like to thank my Thesis Committee Members: Dr. Linda VanAelst, Dr. Wei-Xing Zong, Dr. Stefan Tafrov, and Dr. Nevenka Dimitrova for their advice and comments. They have helped me reach my goal of graduation on time with their constructive criticism, helpful revisions, and flexibility. Dr. Dimitrova has especially been helpful in preparing me for presentations, constantly challenging me in the lab to draw my own conclusions, and reminding me to take time to be a mom too.

My sincere gratitude goes to my advisor, Dr. Robert Lucito, without which this project would not be possible. After stumbling early in my graduate career, Dr. Lucito was there to pick me and get me moving on my current project. We never looked back. Working with Rob has been a stepping stone of my career. He has such a positive and carefree attitude that is easy to get along with in the lab. He also drives for perfection which has helped shape the scientist I am today. And on a personal note, he has been more than understanding of my role as a new parent, allowing me time at home with my infant daughter Lily when I needed it most.

There is not enough room to write the thank you list to the family, friends and lab members who have helped me get to where I am today. First, and foremost, I need to thank my husband Paul. I do not know how I would have gotten through the years without his constant support, encouragement and patience throughout the duration of my graduate studies. I do not know how he survived the last month or so when I was completely stressed out writing my dissertation and taking care of our daughter. Both of which are a full time occupation. He has been an inspiration to keep moving forward. I would not have done it without him. Next, the technicians who have come and gone

from our lab, but most current, I need to mention Mike Vigliotti. Without his support during my maternity and while writing my dissertation this would not be possible. He has helped me in my project and enabled me to take care of my daughter while writing from home. He is a bright and meticulous technician and I cannot express enough thanks to him. Additionally, I would like to thank the fellow graduate student in the lab, Elena Lum and past members of the Lucito lab: Theresa Auletta, James Byrnes, and Christina Hutter. They were there for me when I needed advice, support or reagents, or just to relax and unwind. It was a privilege to work with them. And of course I need to thank Gregg Evans, our technology assistant. He has fixed every problem I have ever had on both my personal and lab computer (which is sometimes a daily event). He is a dear friend and colleague.

In addition, I dedicate my graduate career to Dr. Betsy Sutherland. She encouraged me to pursue a career in biology and to further my education to obtain a Ph.D. She was a mentor to me early in my career. Her guidance and spirit has never left me. She would have been here today to support me in my defense. It was an honor and privilege to know her. I hope I make her proud! May she rest in peace.

Finally, I would also like to dedicate my work to my nephew Louis who died of cancer in 2006 at only 18 months. His battle with cancer has been the pavement for my work. He continues to be my angel and a constant inspiration to my research.

Chapter I: Introduction

1.1 Summary

Cancer is the abnormal, uncontrolled growth of previously normal cells. The transformation of a cell results from alterations to its DNA that accumulate over time, which no longer allow the cell to function properly. Tumors can either be benign (localized, noninvasive) or malignant (invasive, metastatic). While no single genetic defect is causal, the development of cancer implicates a loss in homeostatic control leading to the aberrant growth of cells, invasion of normal tissue, and cell dissemination to secondary sites [1]. At a molecular level, the involvement of tumor suppressors and proto-oncogenes act as a system of checks and balance for the cell and ultimately determine whether a cell will proliferate or undergo apoptosis. Aberrant changes in the genetic and epigenetic information of the cell cause it to no longer function properly leading to the aberrant activation of signaling pathways [2].

Chemoresistance of tumor cells is recognized as the primary cause of failure of chemotherapeutic treatment of most human cancers [3]. Despite numerous reports, understanding the mechanism of chemoresistance remains a challenge in studying human tumors. Cellular factors play a major role in chemoresistance in several tumors, including lung, breast, pancreatic, and ovarian [4-6]. Resistance of tumor cells to chemotherapy involves critical pathways but they are still relatively unknown. Chemoresistance is most evident in metastatic disease (stage 3 and stage 4). A detailed

understanding of the mechanisms involved will provide better clinical approaches to treating tumors [7].

This dissertation delves into understanding the role of chemoresistance in epithelial ovarian cancer (EOC). As there are no overt symptoms associated with its onset and no current reliable methods for early detection, most ovarian cancers are not discovered until they have already metastasized. Generally, the current regime of chemotherapy for EOC consists of a combination of platinum based drugs and paclitaxel [8]. Although these compounds have improved treatment success rates over the past decade, the majority of patients experience a relapse, and in most patients, the disease persists [9].

The main objective of this project was to study platinum resistance in EOC integrating several different approaches. Since therapy response can be predicted by the clinical variable of platinum sensitivity, we used this indicator to identify targets important to treatment [10]. Since there are few alternative treatments available for patients with ovarian cancer it is important to understand the molecular mechanisms of platinum resistance so therapeutics that are better, or therapeutics used in conjunction with platinum can be developed. In addition, platinum based treatments are administered to patients with other tumor types, such as colon, non-small cell lung, bladder, and several others, and the mechanism we dissect for ovarian cancer are likely to be applicable to these other tumor types.

By studying DNA methylation we have identified genes repressed in resistant patients and gained a better understanding of therapy response. Furthermore, we have identified *CHD3* as a novel player in platinum-based resistance in ovarian cancer cell

lines and primary ovarian tumors and we go on to define a molecular mechanism possibly involved. *CHD3* is a member of the Mi-2 NuRD complex associated with transcriptional repression [11]. This protein is one of the components of a histone deacetylase complex which participates in the remodeling of chromatin by deacetylating histones [12]. Chromatin remodeling is essential for many processes including transcription. A role for CHD3 in chemoresistance has not been examined.

1.2 Ovarian cancer overview

Ovarian cancer is the deadliest cancer in the female reproductive tract due to the nonspecificity of symptoms and lack of early diagnostic tests [13, 14]. Tumors that develop in the ovaries are named according to one of three types of cells from which the cancer originates: germ, stromal, and epithelial [15]. Germ cell tumors are found in the cells that produce the eggs inside the ovary. Stromal cells are located between the germ cells and produce most of the female hormones (see **Table 1-1**). Epithelial ovarian cancer is the most common cancers in women and the leading cause of death from gynecological malignancies with an estimated 14,600 deaths in 2009 in the United States alone. Epithelial ovarian neoplasms are notoriously heterogeneous, with over 90% of all malignant ovarian tumors being epithelial in type [16]. EOC is thought to arise directly from the surface epithelium of the ovary and its associated inclusion cysts or indirectly from benign ovarian lesions derived from the inclusion cysts [17]. Additionally, recent research suggests that the epithelia of fallopian tubes can become a tumor and then spread

into both the ovaries and the abdomen [18]. Epithelial ovarian cancers can be further classified into five histological subtypes: high and low grade serous, endometrioid, mucinous, clear cell, and undifferentiated [19] and of these serous is the most common (**see Table 1-1**).

The initiating genetic events in EOC are poorly understood but are thought to arise from a succession of alterations involving oncogenes and tumor suppressor genes which have a critical role in normal cell growth regulation. This succession of events ultimately results in different patterns of expression such as amplification and deletion. In the past three decades much progress has been made in understanding the pathogenesis of EOC however the low 5-year survival rate has not significantly changed for stage III and stage IV EOC [13]. In contrast, patients with stages I and II disease have 5-year relative survival rates greater than 89.3% [20]. Most patients initially respond to chemotherapy but the tumor frequently will return with acquired resistance. Because there is no reliable method or predictive indicators to identify early symptoms, EOC is frequently diagnosed after it has already metastasized, and unfortunately approximately 25% of patients present with resistance to chemotherapy and high frequency of reoccurrence. Therefore, women diagnosed with advanced-stage EOC and have 5-year survival rates rarely exceeds 30% [8, 21]. The source of recurrences and lack of response to chemotherapy is currently unknown (**see Table 1-2**).

A number of genetic alterations are frequently encountered during ovarian tumorigenesis, including oncogenic activities of *KRAS*, *BRAF*, *MYC* and *AKT*, and silencing mutations of *TP53*, *RB*, and *PTEN*. Activation of several oncogenes has been reported in subsets of ovarian cancers. Abnormalities in tyrosine kinases, nonreceptor

tyrosine kinases, G proteins, and transcription factors have been observed. Increased activity of membrane PI3 kinase and AKT kinase can inhibit apoptosis by phosphorylating BAD and/or caspase 9. *AKT2* is amplified in 12% ovarian cancers and associated with poorly differentiated histology [21]. The GTP binding protein encoded by ras is mutated in 90% pancreatic cancers and in a majority of lung and colon cancers. However, ras mutations are inconsistent in EOC and activation of the Ras protein has been observed in the absence of mutation in a majority of ovarian cancer cell lines (Patton, SE 1998). *BRAF* mutations are thought to be early events in the neoplastic transformation of low grade serous tumors [22]. Expression of Myc protein is increased in 30% of ovarian cancers [23, 24]. However, this overexpression does not correlate with prognosis, as is the case with several other oncogenes. Furthermore, aberrant signaling of growth factors such as TGF-β can increase invasiveness of ovarian cancers related to the induction of protease activity [19].

Among the tumor suppressor genes, loss of functional *p53* and loss of *ARHI* expression appear to be most prevalent [25]. *ARHI* is a RAS/RAP homolog that induces p21 WAF/CIP1, downregulates expression of cyclin D1, truncates signaling through RAS/MAP and inhibits the growth of cancer cells that lack its expression [26].

Germline mutations of *BRCA1* and *BRCA2* tumor suppressor genes are responsible for 90%-95% of hereditary ovarian cancers [27]. The lifetime risk of developing ovarian cancer is about 20%–50% in patients carrying *BRCA* mutations [27]. Both genes play integral roles in genomic stability and integrity, cell cycle control, and apoptosis. Specifically, both genes are essential for DNA repair and activation of cell

cycle checkpoint control. Repair-deficient cells (without *BRCA* genes) accumulate chromosomal abnormalities with loss of cell cycle control [28] (**see Table 1-3**).

1.3 Chemotherapy

Researchers face a number of major challenges in eradicating cancer. These include discovering new genes that play a causal role in neoplasia, understanding how multiple genetic mutations interact to produce cancer, and finally, developing new therapeutics that will target cancer by exploiting the genetic changes unique to cancer cells [29].

Cancer chemotherapy began in the early 1940s with the use of nitrogen mustards and folic acid as antagonist drugs. Since then, the introduction of several agents for chemotherapy has emerged. These include compounds such as taxanes, camptothecins, platinum based agents, nitrosoreas, and anthracyclines/epipodophyllotoxins [30]. Paclitaxel (taxol), a taxane, is a mitotic inhibitor which directly affect microtubule assembly during anaphase in the cell cycle [31]. Alkylating agents such as the platinum based drugs have the ability to alkylate many nucleophilic functional groups in a cell. They impair cell function by forming covalent bonds with the amino, carboxyl, sulfhydral, and phosphate groups in a cell [32]. Cisplatin and carboplatin in combination with a taxane are common chemotherapy drugs used in the treatment of ovarian cancer (see **Table 1.4**).

1.4 Mechanisms of drug resistance

It is generally accepted that EOC's insensitivity to conventional platinum-based chemotherapy tends to be followed by a poor prognosis. Drug resistance is an important factor in the poor prognosis of patients with EOC. Thus, chemoresistance can be viewed as the final step in tumor progression and is mainly responsible for the majority of ovarian cancer-related deaths. EOC can be classified by both de novo resistance and acquired resistance [16]. Approximately, 20% of advanced-stage ovarian tumors do not respond to chemotherapy initially, and are considered to have de novo resistance. Those tumors that respond initially and later recur are considered to acquire resistance as a result of the selection of drug-resistant clones during treatment with chemotherapy. Much work has been done to identify gene signatures associated with resistance to chemotherapy, however there has been little success [33-36]. This suggests that some of the genes in these signatures may be secondary phenomena rather than the primary process associated with chemoresistance. Several mechanisms involved in drug resistance have been proposed, including drug inactivation, decreased drug accumulation, increased drug detoxification, and increased DNA repair activity and tolerance [37].

1.4.1 Drug efflux

Adenosine 5′-triphosphate (ATP)-binding cassette (ABC) transporters, such as ABCB1 (also known as P-glycoprotein), ABCC1 (also known as multidrug resistance associated protein-1) and lung resistance protein, are known to affect intracellular drug concentrations and are important multidrug resistance factors [38]. ABCB1 and ABCC1 function as an ATP-dependent efflux pump for cytotoxic drugs, such as paclitaxel (PTX) and platinum agents [38, 39]. Overexpression of these genes mRNA in certain tumors is

an indicator of poor prognosis in patients treated with chemotherapy. However, their role in EOC is unclear, and several studies show conflicting expression results and association with chemoresistance. Most conclusions suggest that these multidrug resistance proteins are not contributing factors to chemoresistance in EOC [40].

1.4.2 Drug inactivation

Several drug detoxification systems can also diminish the amount of intracellular drug activity. Cellular detoxification via the glutathione system is known to be involved in the metabolism of various cytotoxic agents, including platinum agents [39, 41]. Specifically in EOC, the pathways that mediate drug resistance include increased levels of glutathione (GSH), glutathione S-transferases (GSTs), and metallothioneins (MT). GSTs are a multigene family whose members catalyze the conjugation of glutathione with electrophilic drugs. They have been implicated in resistance to several agents, including platinum drugs [42]. However, studies have shown a minimal potentiation of cisplatin and other platinum drug sensitivities in cell lines treated with these systems [43-45]. MT proteins have high cysteine content and therefore have been suggested to inactivate electrophillic anticancer drugs.

1.4.3 Increased DNA repair

Nucleotide excision repair (NER) is a multienzyme DNA repair pathway in eukaryotes that has been implicated in drug resistance in human tumor cells. Once

platinum drugs form cytotoxic DNA adducts, cells must either repair or tolerate the damage in order to survive. NER removes DNA damage as part of an oligonucleiotide 24-32 residues long. The capacity to rapidly and efficiently repair DNA damage clearly plays a role in determining a tumor cell's sensitivity to platinum drugs and other DNA damaging agents. For example, cell lines deficient in their ability to repair platinum-DNA adducts such as testicular nonseminomatous germ cell tumors are unusually sensitive to cisplatin [46]. Similarly, DNA repair capacity differences can be seen in non-small cell lung cancer as compared to small cell lung cancer [47]. Furthermore, increased DNA repair of platinum DNA lesions in cisplatin resistant cell lines as compared to their sensitive counterparts has been shown in several human cancer cell lines including ovarian ([48] breast [49], and glioma [50]. Expression levels of *ERCC1* and *XPA* genes have been shown to be higher in malignant tissue from ovarian cancer patients resistant to platinum chemotherapy as compared to those who are responsive [51].

1.4.4 Increased DNA damage tolerance

DNA mismatch repair systems (MMR), which correct errors occurring during DNA replication, plays a critical role in DNA damage tolerance in the cell and therefore genomic stability [52]. The MMR system has been observed to be dysfunctional in cisplatin resistant cells. It has been reported that loss of MMR is associated with low level cisplatin resistance, and that selection of cells in culture for resistance to this drug often results in cell lines with loss of functional MMR [53]. For example, the human

colon cancer cell line HCT116 is MMR deficient because of a deletion in one allele of *hMLH1* and a mutation in the other copy. This cell line is resistant to both cisplatin and carboplatin, relative to MMR proficient cell lines [54]. Furthermore, studies suggest that MMR may function as a DNA damage sensor, triggering a programmed cell death pathway, rendering cells with intact MMR more sensitive to DNA damage [54, 55]. This in turn creates an environment that promotes the accumulation of mutations in drug sensitivity genes.

1.4.5 Aberrant cell cycle

Cell proliferation depends on the ability of the cell to successfully pass through the G1, S, G2, and M phases of the cell cycle. This cell progression is controlled by cyclin dependent kinases (CDKs), which are regulated by cyclin binding, phosphorylation, and CDK inhibitors (p16, p21, and p27). Following drug treatment cell arrest takes place and elevated levels and activity of p53, a known tumor suppressor protein, is seen. p53 mutations and expression changes in EOC is a common event [55]. When p53 is mutated, cells are less prone to initiate apoptosis and more resistant to DNA damage by chemotherapeutic drugs. Although many studies try to associate p53 status with platinum sensitivity, many of the results are conflicting and therefore, p53 status is not a likely indicator for determining cell survival. Furthermore, increased expression of the cdk inhibitor p21 waf/cip1 allows for a cell to continue through the cell cycle without allowing time for the cells to repair DNA damage. This is evidenced by studies that show that p21 deficient cells are hypersensitive to DNA damaging agents [56].

Furthermore, cytotoxic drugs are primarily effective against proliferating cells; therefore, quiescent cells show a degree of drug resistance relative to cycling cells [56]. One emerging model for the development of drug-resistant tumors invokes a pool of self-renewing malignant progenitors known as cancer stem cells (CSCs) or cancer-initiating cells (CIC) [57]. According to the CSC hypothesis, the cancer cells are resistant to chemotherapy because of their stem cell properties specifically lack of proliferation. Chemoresistance arises due to the presence of a small pool of slowly cycling cells that are intrinsically resistant to chemotherapy or develop mutations and are selected during the course of chemotherapy [58].

1.4.6 Apoptosis inhibition

The sensitivity of tumor cells to platinum drugs and other anticancer agents may also be dependent upon the ability of the cancer cell to undergo drug-induced apoptosis. For example, the bcl-2 gene family, which encodes a group of pro and antiapoptotic proteins that regulate mitochondrial function and intracellular calcium concentration, function as a cell survival/cell death regulator by forming homo and heterodimers with each other. Studies show that overexpression of bcl-2 or bcl-XL prevents the disruption of the mitochondrial transmembrane potential and prolongs cell survival in some cells following exposure to cisplatin and other anticancer drugs [59]. Aberrant regulation of several key apoptotic regulators, including p53, the Inhibitor of Apoptosis (IAP) family, the Akt family, and the death-receptor family, may influence the response of ovarian cancer cells to cisplatin [60]. For example, cisplatin has been shown to upregulate the

pro-apoptotic factors p53, Fas, and Bax in a number of cell types [61-63]. However, it also down-regulates specific cell survival factors such as Xiap and Akt [64]. Recent evidence suggests that chemoresistance may represent an overall imbalance between these two phenomena [65].

1.4.7 Cytoskeleton organization

The cytoskeleton is a cellular dynamic structure capable of maintaining cell plasticity, and it is responsible for constant changes within the cell setting including cell migration, cell division, and intracellular trafficking. Because microtubules are so dynamic and involved in many different cellular functions, anticancer drugs such as taxanxes and vinca alkaloids are often designed to target them [3]. Antimicrotubule resistance develops through several mechanisms including MDR, alterations of microtubule components, and deficient apoptotic signaling through p53, bcl-2, or bcl-xl [66]. Additionally, EMT is another cytoskeletal change that can confer resistance to chemotherapy drugs [67-69]. EMT is classified as a unique process by which epithelial cells undergo remarkable morphologic changes characterized by a transition from epithelial cobblestone phenotype to elongated fibroblastic phenotype (mesenchymal phenotype) leading to increased motility and invasion [70]. Specifically, in resistant EOC cells, enhanced mitogen activated protein kinase (MAPK) and Akt phosphorylation and cell proliferation can be seen. Moreover, these cells the expression of E-cadherin transcriptional repressors, including Snail, Slug, and Twist, as well as of mesenchymal markers, such as Vimentin and N-cadherin, were upregulated and linked with enhanced

invasive behavior [68]. A summary of chemoresistant mechanisms is outlined in **Table 1.6**.

1.5 Ovarian cancer platinum therapy

Efforts to treat ovarian cancer include a very large collection of conventional chemotherapy agents and growing lists of molecular targeted agents that occasionally provide tumor cytoreduction and clinical benefit [71]. While initially responsive to chemotherapy, the majority of patients experience tumor reoccurrence, with poor survival primarily attributed to the accumulation of residual clonal cells that either acquire or possess intrinsic drug resistance [72]. Most women with this diagnosis will die as a result of their disease despite cytotoxic chemotherapy. Therefore, the search for the molecular basis of this resistance is essential for the successful treatment of the disease. Currently platinum-based chemotherapy (such as cisplatin or carboplatin), coupled with a taxane based drug such as paclitaxel is the primary treatment for ovarian cancer and is also used in a wide variety of other malignancies [73]. Platinum cytotoxicity results from the formation of DNA monoadducts and crosslinks, which, in turn, promote the development of apoptosis inducing double strand breaks during replication [74, 75]. Paclitaxel is a mitotic inhibitor that works by stabilizing microtubules and as a result, interferes with the normal breakdown of microtubules during cell division. It is not completely clear as to exactly why cell death occurs after platinum and paclitaxel treatment. It is clear that platinum DNA binding results in the inhibition of DNA replication and transcription, but whether these effects are sufficient to cause cell death at clinically achievable platinum

doses through DNA damage and depletion of homeostatic enzymes is uncertain. As for taxane drugs such as paclitaxel, the stabilization of tubulin results in aberrant mitosis, which is also destructive to DNA [76]. The current hypothesis states that cell death results due to DNA damage which activates programmed cell death pathways. However, it is unknown whether the DNA adducts formed by platinum drugs are sufficient for cell cytotoxicity. Although these lesions will interfere with DNA replication, its results on naked DNA verse the cell as a whole, may be different for both cisplatin and carboplatin treatment, even though the active form of both drugs is identical [77]

1.6 Ovarian cancer platinum resistance

As stated, platinum treatment on its own produces only a modest clinical tumor response and attributes to tumor resistance [78]. This platinum based chemoresistance almost always occurs in recurrent patients and some patients are intrinsically resistant to treatment. Thus, it is critical to identify biomarkers that can predict patient response to platinum drugs so that optimal treatment can be administered immediately. While mutation of individual genes such as *RAS* or *p53* can be relatively common in a tumor type, overall there is considerable heterogeneity between the genetic, epigenetic and pre- and post-translational alterations in the tumor cells of one patient to another patient [79].

In OC, *CCNE1* (cyclin E) amplification has been associated with an overall poor survival in ovarian carcinoma [80] and a recent finding suggests that this amplification is also associated with chemoresistance [37]. This suggests *CCNE1* may have utility as a tumor marker and may also have a role in chemoresistance. Enhanced DNA repair

ability of tumor cells prevents the accumulation of lethal DNA damage from cytoxic agents [34]. DNA DSB repair involves pathways of homologous recombination and nonhomologous end joining [81]. The Me11/Rad50/Nbs1 (MRN) complex plays a major role in both these pathways and in driving chemoresistance [82].

Tumor suppressor *BRCA1* and *BRCA2* are frequently mutated in familial ovarian cancer and these ovarian carcinomas are generally sensitive to platinum compounds [83]. However, over time the majority of these *BRCA1/2*-deficient cancers become resistant and patients die from refractory diseases. The mechanisms of platinum resistance in *BRCA1*-mutated ovarian cancer remain unclear. Because both *BRCA1* and *BRCA2* are involved in homologous recombination DNA repair [84], Swisher et al. [85] hypothesized that secondary mutations of the mutated *BRCA1* gene mediate acquired resistance to platinum in *BRCA1*-mutated ovarian cancer. Knowledge of the molecular pathways involved in chemotherapy resistance still remains to be elucidated. Genes hypothesized to be involved in chemoresistance of several cancers are listed in **Table 1.5.**

1.7 Methylation and cancer overview

DNA methylation is an important epigenetic mechanisms that control gene expression, chromatin structure, genome stability and X chromosome inactivation [86]; [87]. DNA methylation involves the chemical modification of cytosine by the addition of a methyl group to the 5′ carbon of the cytosine base in CG dinucleotides. Abnormality in DNA methylation can lead to changes in gene expression and serious imbalance in normal function of cells (**see Figure 1.1**). In turn, this promotes pathological conditions.

In particular, a substantial change in DNA methylation is often seen in the genome of cancer cells [87]. This aberrant methylation includes genes involved in control of DNA repair [88], cell cycle control [89], signal transduction [90], angiogenesis [91], invasion [92] and drug sensitivity. Gene-promoter specific hypermethylation of tumor suppressor genes is frequently seen in genes such as *RB, CDKN2A, VHL, APC, MLH1, RASSF1A* and *BRCA1* [93-96] (**see Table 1.7**).

Early changes in gene expression are a function of aberrant methylation and research has shown that EOC is heterogeneous having multiple genetic and epigenetic changes [97]. Most studies to date have focused on candidate gene approaches to identify epigenetically regulated genes in ovarian cancer, in particular, methylated and silenced candidate tumor suppressor genes. Selected targets include genes with downregulated expression in ovarian cancer, genes in regions with known loss of heterozygosity (LOH) in ovarian cancer and thus where tumor suppressors likely reside, and genes that have been shown to be epigenetically regulated in other cancers.

We hypothesize that the epigenome of the cancer cell likely underlies at least a component of intrinsic and acquired chemoresistance. Aberrant hypermethylation in the ovarian cancer genome inactivates tumor suppressors which result in the loss of function of key genes (*e.g. BRCA1*) or the impairment of key pathways (such as BRCA/HR) Several studies reported that *BRCA1* was hypermethylated in 10–15% of sporadic ovarian tumors and that hypermethylation was strongly associated with loss of expression at the RNA or protein level [98]. It was also reported that patients with hypermethylation of *BRCA1* in ovarian tumors had a better survival [98]. The loss of *BRCA1* expression caused by promoter hypermethylation will disrupt BRCA-associated DNA repair and

may sensitize tumors to BRCA-directed therapies, such as PARP inhibitor therapy. Additionally, several growth inhibitory genes are silenced by methylation in cancer including *ARHI, RASSF1, DLEC1* and *OPCML* [99]. ARHI is a ras superfamily tumor suppressor and is associated with loss of expression as well as LOH in 40% of ovarian carcinoma. DLEC1 (deleted in lung and esophageal cancer 1) is a putative tumor suppressor in lung and other cancers found in a region of frequent LOH in cancer. *DLEC1* expression is downregulated in ovarian cancer cell lines and primary invasive epithelial ovarian cancers, where its expression is correlated with hypermethylation of the DLEC1 promoter [100]. p16 (CDKN2A) encodes a cyclin-dependant kinase inhibitor involved in the regulation of the cell cycle. p16 expression is frequently disrupted in cancer and its locus is found in a region of frequent LOH in cancer, including ovarian carcinoma [101]. Specifically, hypermethylation of the p16 promoter region is important in a subset of human carcinomas including lung, head and neck, and pancreatic cancer [102, 103], but it is unclear whether p16 methylation plays a role in ovarian carcinogenesis [101]. The *RASSF1A* tumor suppressor gene is one of the most frequently detected epigenetic events in human cancers and is methylated and silenced in 10–50% of primary ovarian cancers [104]. Global DNA hypomethylation increases with malignancy in ovarian epithelial neoplasms [105]. There are, however, limited examples of specific gene activation by hypomethylation in ovarian cancer. For example, demethylation of the maspin (SERPINB5) promoter in ovarian cancer cells is associated with a gain of maspin mRNA expression [106]. Abnormal expression of the metastasis-related gene synuclein-γ (SNCG), a member of a family of small cytoplasmic proteins, is hypomethylated and

reexpressed in aggressive ovarian cancer cell lines [105] and in a substantial proportion of malignant ovarian carcinoma samples [107].

1.7.1 Methylation and chemoresistance

Epigenetic inactivation of genes is crucial for control of normal cell growth and is a hallmark of cancer cells [1]. These epigenetic mechanisms include DNA methylation, histone modification and other components of higher order structure leading to regulation of gene transcription. Epigenetic gene regulation has the potential to affect both intrinsic and acquired drug resistance [108] and therefore epigenetic markers may prove useful in predicting chemotherapy response and outcome in patients with ovarian cancer. Methylation is thought to influence several processes, such as DNA repair and damage response pathways, cell cycle control, and apoptosis which are thought to be implicated in drug resistance [109]. For example, Teodoridis et al. [104] showed that methylation of at least one of the three genes involved in DNA repair/drug detoxification (*BRCA1*, *GSTP1*, and *MGMT*) is associated with improved response to chemotherapy of patients with late-stage epithelial ovarian tumors. Chemotherapy can act as a positive selector on subpopulations of cells in an initially chemoresponsive tumor. A direct role for epigenetic inactivation genes underlying acquired chemoresistance at disease relapse has been demonstrated in a number of recent studies [110]. CpG methylation of *MLH1* mismatch repair gene has been associated with platinum resistance in relapsed invasive ovarian cancers and poor overall survival [111]. In recent work, Zhang et al. examined hypermethylation and inactivation of *RUNX3* in ovarian cancer tissues and cell lines that

may correlate with early events related to its repression [112]. Additionally, Staub *et al*. provide evidence of CpG methylation as a mechanism of *HSulf-1* inactivation in chemoresistance of ovarian cancer cell lines. Furthermore, they suggest the use of 5-aza-2'-deoxycytidine to increase transcription of this gene [113]. Ongoing genome-wide studies e.g., The Cancer Genome Atlas (TCGA) should reveal the methylation status of other genes in ovarian cancer. We hypothesize that this methylation is in part responsible for the onset of chemoresistance of EOC.

1.8 Chd family of chromatin remodelers

Dynamic regulation of chromatin structure is of fundamental importance for modulating genomic activities such as transcription, recombination, repair, and replication in eukaryotes. Chromatin remodeling enzymes play a critical role in this process. Members of the Chd protein family are enzymes that belong to the SNF2 superfamily of ATP-dependent chromatin remodelers (see **figure 1.2**). These enzymes can be further divided into two classes: those that mediate post-translational histone modifications and those that alter the histone-DNA contacts using ATP [11]. Chd proteins are important regulators of transcription and recent work has demonstrated their roles in both development and disease. Despite common features, such as their chromodomain and helicase domain, CHD proteins have been described as having multiple roles and interacting partners [114]. Therefore, they are thought to play diverse roles in both transcriptional activation and repression.

1.8.1 Chromodomain-helicase-DNA-binding protein 3

Members of the *CHD3* subfamily are characterized by the presence of two tandemly arranged chromodomains as well as the presence of one or two PhD (plant-homeo-domain) zinc fingers preceding the chromodomains [115]. In animals, *CHD3*-class proteins exist in diverse multiprotein complexes including the Mi-2/NuRD (nucleosome remodeling and histone deacetylation) complex, which involve histone deacetylase and the dMEC complex. dMEC represses target gene expression in a histone deacetylation-independent manner, although some *CHD3*-class proteins exist as a monomer [116]. This multiprotein complex is implicated to couple ATP-dependent chromatin remodeling and histone deacetylation resulting in transcriptional repression [117]. Several studies implicate a function of *CHD3* family members in transcriptional activation [94, 118].

CHD3 protein is part of the PICKLE (PKL) and PICKLE RELATED2 (PKR2) in plants and is required for the expression of many genes that are repressed by Polycomb Group (PcG) proteins [11]. PcG proteins are required to maintain pluripotency, prevent cell differentiation in mammals, and are important in ensuring that the correct expression of specific transcriptional programs at defined developmental stages. This takes place by their default silencing activity. This activity is often counteracted by trxG proteins that activate PcG target genes and prevent PcG mediated silencing activities. Recent results show that *CHD3* has trxG-like functions in plants and is required for the expression of many genes that are repressed by PcG proteins [6]. The exact role of PcG proteins in tumorigenesis is still unclear, but they are known to play a role in chromatin remodeling

[119]. Furthermore, *CHD3* class of chromatin remodeling factors could also be recruited to chromatin by interaction with cell type-specific transcription factors, including BCL-6, Hunchback and Tramtrack 69 [120] and regulate target genes of these transcription factors in animals. These complexes may play important roles in regulating invasive growth pathway in breast cancer as well as regulating cell fate during B lymphocyte differentiation [115, 116].

The *CHD3*/CHD4 family of CHD proteins is thought to play important roles in not only transcription but in replication-coupled chromatin assembly, and in DNA repair. *CHD3* and Chd4 function is usually associated with transcriptional repression [121]. *hCHD3* appears to be ubiquitously expressed in several tissues [122]. In mouse neonatal tissues, *mChd4* mRNA is expressed at high levels in the thymus, the kidney, in specific areas of the brain, in hematopoietic foci in the liver, in hair follicles, and in mucosal epithelia [123]. Similar patterns of expression for *mCHD3* mRNA were detected in these tissues but at lower levels than *mChd4*, as judged by in situ hybridization [123].

1.8.2 Role for Chd in human disease

Mutations in genes encoding SNF2-like enzymes are known to cause a spectrum of disease phenotypes. The identification and characterization of these enzymes is critical for understanding the genetic events underlying the progression of disease. To date, *hCHD3-hCHD5* and *hCHD7* have been implicated in human disease processes [124]. For example, autoantibodies against *CHD3* are found in a subset of patients with dermatomyositis, which is a connective tissue muscle disease associated with

inflammation. The etiology of dermatomyositis is poorly understood, but a correlation between this disease and an elevated incidence of cancer has been established [125]. More recently, *hCHD3* was associated with Hodgkin's lymphoma. A yeast two-hybrid screen identified an interaction between h*CHD3* and Ki-1/57, an intracellular phospho-protein, and between *hCHD3* and CGI-55, a mRNA-binding protein [126, 127]. Although the functions of these protein is unknown, Ki-1/57 specifically detects the malignant cells in Hodgkin's lymphoma [128]. *CHD5* expression is largely confined to neural tissue, where its associations are not well characterized. A recent functional study identified CHD5 to have tumor suppressive functions in tumors of breast, colon, and neuroectodermal origin [129]. The chromodomain sequence of subfamily II (*e.g.* CHD3) differs significantly from that of either subfamily I or III (*e.g*. CHD5), making it unlikely for them to interact with histone tails [130]. In addition, biochemical studies have demonstrated that Drosophila Mi-2 is able to interact directly with “tailless” nucleosomes as well as with DNA under low-salt conditions.

The physiological roles of CHD proteins in disease, specifically human cancers has been of interest to many researchers Recent publications suggest that *CHD3* is upregulated in pancreatic, gastric and colon cancers. However, a functional role for *CHD3* in cancer has not been examined and specifically the role that this gene plays in ovarian cancer has not been elucidated.

1.9 Summary

A better understanding of the basic biology of epithelial ovarian cancer is crucial to treatment options. It is apparent that in model systems of platinum resistance, many mechanisms appear to participate. Since there are few alternative treatments for patients with ovarian cancer it is important to understand the molecular mechanisms of platinum resistance. Ultimately, this will lead to better therapy treatments either as single agents or used synergistically with other agents. Furthermore, platinum based treatments are administered to patients with other tumor types, such as colon, non-small cell lung, bladder, and several others, and the mechanism we dissect for ovarian cancer are likely to be applicable to these other tumor types [131].

The main goal of the dissertation was based on the hypothesis that EOC will take advantage of epigenetic changes to become resistant to therapy. Therefore a genome wide screen of DNA methylation to identify those genes that segregate tumors based on resistance and sensitivity. The first aim of this dissertation was to test the most significant genes in an in-vitro chemoresistant functional RNAi screen. The second aim of the dissertation was to functionally characterize the effect that decrease of the CHD3 protein, one of the gene hits, has on cellular resistance to platinum. Finally, the last aim on this study was to elucidate a possible mechanism for chemoresistant phenotype in vitro. Each of these aims and the novel contributions we have made in this study will be further discussed in the upcoming chapters.

We find that cells containing the small hairpin RNA (shRNA) targeting CHD3, a chromo domain protein, has a unique slow growth and invasive phenotype. Taken

together, we demonstrate *CHD3* is epigenetically regulated gene that is transcriptionally repressed and plays an important role in platinum-based resistance in ovarian cancer cell lines and primary ovarian tumors. Furthermore, our data demonstrates that the development of platinum resistance in EOC cells is accompanied by a transition from epithelial to a mesenchymal phenotype. Therefore, we provide the evidence for a possible mechanism of resistance in this disease through the silencing of a chromodomain protein which facilitates an epithelial mesenchymal transition. In conclusion, we predict *CHD3* to be an important mediator of the response to platinum chemotherapy.

Figure 1-1

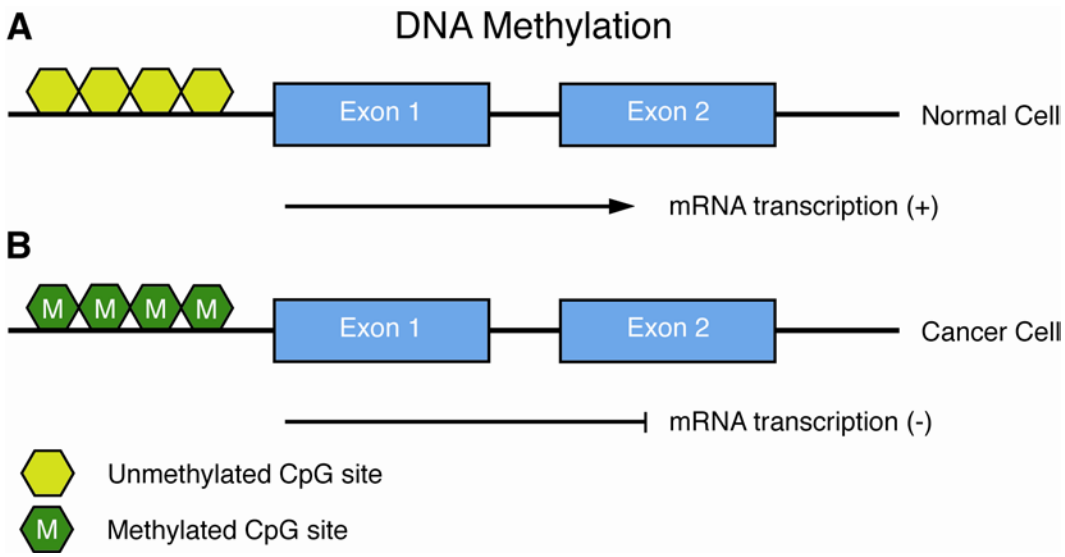


Figure 1-1 DNA methylation is a heritable epigenetic modification process that occurs in some eukaryotes whereby CpG dinucleotides are methylated at the C5 position of cytosine. The methylation of the 5’ regulatory regions of genes results in gene silencing. In humans and most mammals, DNA methylation is the only known natural modification of DNA, and only affects the Cytosine base (C) when it is followed by a Guanine (G). Thus, in these organisms, DNA methylation only occurs at CpG sites. Between 70-80% of all CpGs are methylated. Unmethylated CpGs are grouped in clusters called "CpG islands" that are present in the 5' regulatory regions of many genes. In many disease processes such as cancer, gene promoter CpG islands acquire abnormal hypermethylation, which results in heritable transcriptional silencing. We hypothesize that tumor cells take advantage of this epigenetic mechanism to become resistant.

Figure 1-2

CHD protein homology

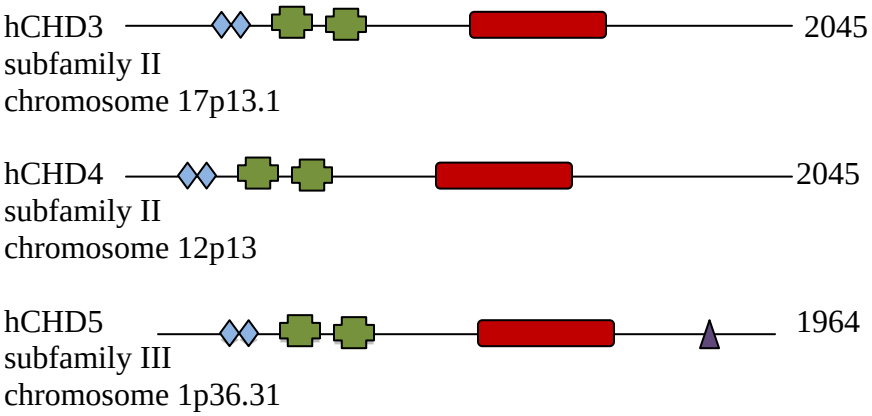


Figure 1-2 is a schematic representation of protein domains found within human Chd genes of subfamily II and III. Chromodomains (green cross) and a SNF2-like ATPase domain (red rectangle). As you can see from the diagram, CHD3 and its closest family member CHD4 share several common domains. Additional domains have also been predicted for several members of this subfamily, including paired PHD Zn-finger-like domains (blue diamond), SANT domains (purple triangle). Additionally, the subfamily, chromosome location and sequence information is listed as obtained from NCBI [121].

Table 1-1

Types of Ovarian Cancer
Surface Epithelial Tumors
Serous Tumors
Mucinous Tumors
Endometrial Tumors
Clear Cell Carcinomas
Malignant Mixed Mullerian Tumors
Transitional Cell Tumors
Small Cell Carcinomas
Sex Cord Stromal Tumors
Granulosa Cell Tumors
Fibromas and Thecomas
Sertoli Cell Tumors
Sex Cord Tumors
Gonadoblastomas
Leydig Cell and Steroid Cell Tumors
Germ Cell Tumors
Dysgerminomas
Yolk Sac Tumors
Embryonal Carcinoma
Choriocarcinoma
Immature Teratoma
Carcinoid

Table 1-1 lists the different types of ovarian cancer. Epithelial cancer makes up the majority of ovarian cancer related deaths [11, 12].

Table 1-2

Stage	*Characteristics*	*Five Year Survival (%)*
I	Growth limited to ovaries	80 to 90%
II	Growth involving one or both ovaries	70 to 80%
III	Tumor involving one or both ovaries with malignant extensions	15 to 40%
IV	Growth involving one or both ovaries with distant metastases	5 to 20%

Table 1-2: Five Year Survival for Ovarian cancer. This table lists the stages of ovarian cancer, characteristics seen with the stages. As you can see from the table, survival of patients with stage 1 or stage 2 ovarian cancers is pretty high, while stage 3 and stage 4 is poor. [7, 13]

Table 1-3

A

Gene	**Chromosome**	**Function**
SPARC	5q31	Matrix
DOC2	5p13	Binds GRB2
PTEN	10q23	Phosphatase
NOEY2	1p31	Induces p21; inhibits cyclin D
p53	17p13	DNA stability; apoptosis
LOT-1	6q25	Zinc finger
OVCA1	17p13	unknown

B

Type	**Example**
Receptor Kinases	Overexpression Her-2
	Mutation EGFR
	Overexpression of MYC
Nonreceptor Kinases	Amplification of PI3 Kinase
	Amplification of AKT kinase
	SRC Kinase Activation
RAS Activation	

Table 1-3 Lists the most notable genes in ovarian cancer. **Panel A** lists the putative tumor suppressor genes while **Panel B** lists the oncogenic players [26, 28, 132-136]

.

Table 1-4

Chemotherapy Drug	***Mechanism of Action***	***Example***	***Cancers***
Mitotic Inhibitors	stop mitosis or inhibit enzymes for cell reproduction	taxanes	breast, lung, myelomas, lymphomas, and leukemia
Alkylating Agent	DNA damaging agent	Carboplatin, cisplatin	lung, breast, ovarian, leukemia
Antimetabolites	interfere with DNA and RNA growth	5-fluorouracil (5-FU), capecitabine, methotrexate, gemcitabine	leukemia, tumors of the breast, ovary, and the intestinal tract
Anti-tumor antibiotics	interfere with enzymes involved in DNA replication	doxorubicin, actinomycin-D, bleomycin, and mitomycin-C	leukemia
Topoisomerase inhibitors	interfere with topoisomerases	topotecan and irinotecan, etoposide	leukemia, as well as lung, ovarian, gastrointestinal

Table 1-4 lists the different types of chemotherapy drugs. In ovarian cancer there is usually a combination therapy given, a taxane and a platinum based drug [137-139]

Table 1-5

Apoptosis Genes	Pumps/Detox	DNA Repair	Proliferation
AKT	ATP7B	ATM	APC C-term
APAF1	GST3b	BRCA1	APC N-term
BAD	MDR1	ERCC1	B-TUBULIN
BAX	MRP1	ERCC2	EGFR
BCL2	MRP2	MLH1	HER2
BCL-x(L)	MRP3	MSH2	HER3
BID	MRP4	MSH6	HER4
MRP5	RAD51	HIF1A	KI67
FAS	MRP6	XPA	
MRP8	XRCC1	P16	
SOD1	XRCC5	P21	
NFkB	XRCC6	P27	
PIK3CA	VEGF	P53	
PTEN			
STAT3			
SURVIVIN			
XIAP			

Table 1-5: Possible genes involved in chemoresistance of ovarian cancer. As you can see there are many hypothesized genes thought to play a role in chemoresistance all ranging from different functional groups [140-143]

Table 1-6

General Mechanism	*Example*
Altered Drug Transport	Increased expression of MDR1 gene product, ATP dependent drug efflux pump
Drug Inactivation	Increase innucleioside deaminase that inactivates nucleioside analogs, increase in gluutathione-S-transferase (GST) detoxification
Altered Drug Target	B-tubulin alterations can prevent paclitaxel binding in cell cycle
Drug Sequestration	LRP vault protein sequesters drugs into cellular compartments
Increased DNA Repair	HMG2 can inihibit repair of platinum induced DNA damage
Increased DNA Damage Tolerance	Inactivatio of apoptotic responses by mutation of p53 or overexpression of Bcl-2

Table 1-6 outlines an overview of the mechanisms tumors use to become resistant to chemotherapy. Listed as well are some specific examples [4, 140, 144].

Table 1-7:

Gene	***Role in Tumor Development***	***Tumor***
RUNX3	Developmental regulator, possible oncogene	Ovarian
APC	Deranged regulation of cell proliferation, cell migration, cell adhesion, cytoskeletal reorganization, and chromosomal stability	Breast, Lung, Esophageal
BRCA1	Implicated in DNA repair and transcription activation	Breast, Ovarian
CDKN2A/p16	Cyclin-dependent kinase inhibitor	Gastrointestinal Tract, Head and Neck, Non-Hodgkin's lymphoma, Lung
DAPK1	Calcium/calmodulin-dependent enzyme that phosphorylates serine/threonine residues on proteins; Suppression of apoptosis	Lung
E-cadherin	Increasing proliferation, invasion, and/or metastasis	Breast, Thyroid, Gastric,
ER	Hormone resistance	Breast , Prostate
GSTP1	Loss of detoxification of active metabolites of several carcinogens	Prostate, Breast, Renal
hMLH1	Defective DNA mismatch repair and gene mutations	Colon, Gastric, Endometrial, Ovarian
MGMT	p53-related gene involved in DNA repair and drug resistance	Lung, Brain
p15	Unrestrained entry of cells into activation and proliferation	Leukemia, Lymphoma, Squamous cell carcinoma, Lung
RASSF1A	Loss of negative regulator control of cell proliferation through inhibition of G1/S-phase progression	Lung, Breast, Ovarian, Kidney, Nasopharyngeal
Rb	Failure to repress the transcription of cellular genes required for DNA replication and cell division	Retinoblastoma , Oligodendroglioma
VHL	Altered RNA stability through and erroneous degradation of RNA-bound proteins	Renal cell cancer

Table 1-7: Genes commonly methylated in human cancers [145, 146]. As you can see from the table, there are several methylated genes that may play a role specifically in ovarian cancer such as RUNX3, BRCA1, hMLH1, RASSF1A [98, 100, 104, 147, 148].

Chapter II: High throughput functional screening of candidate genes affecting platinum resistance using pooled shRNA in vitro

2.1 Introduction

Recent advances in the field of RNA interference (RNAi) have enabled researchers to conduct in depth investigations of gene function. High throughput screens in cultured mammalian cells can now be performed using RNAi libraries such as the Hannon Elledge library [149]. Since their discovery, small RNAs have been implicated in regulation of gene expression, transposon silencing, and heterochromatin formation [150-152]. In the RNAi pathway, double stranded RNA (dsRNA) is processed by an RNaseIII enzyme named Dicer into 21 to 23 nucleotide small interfering RNAs (siRNAs). These products have 5' monophosphate ends and two-nucleotide 3' overhangs [153]. One strand of each siRNA duplex is incorporated into the RNA induced silencing complex (RISC). RNAi has enabled genetic approaches in both cultured mammalian cells and intact animals [154-156].

Exogenously produced short hairpin RNAs (shRNAs) could be used to knockdown a specific gene of interest in mammalian cells. When a gene is silenced by RNAi, production of the target mRNA continues regardless of mRNA degradation and a certain level of translation is expected [157]. Different systems for introduction of a shRNA have been developed to study various aspects of the human genome, such as cancer pathways [151]. Retroviral and lentiviral based systems are available which can work well with cells that are difficult to transfect. Lentiviruses are capable of infecting

both dividing and nondividing cells, which is beneficial for cell types that are extremely slow-growing or nondividing [158]. RNAi has become a valuable tool for studying functional genomics and attacking the molecular vulnerabilities of cancer [159]. Additionally, large-scale loss-of-function studies have been especially helpful to the study of cancer, which is caused by multiple genetic events [6]. This, in turn, allows for a more realistic and accurate model of cancer and creates a way to examine tumor response to chemotherapy.

Gene silencing by RNA interference is a powerful genetic tool to identify genes involved in specific biological processes in model organisms and human cells [160]. It is now possible to perform unbiased genetic screens using cultured human cells. Recently, small-scale siRNA (small interfering RNA) and shRNA (small hairpin RNA) screens in human cells have successfully identified genes that modulate cell growth, apoptosis, chemoresistance, and chemosensitivity [161, 162]. Large scale RNAi screens are useful tools that allow a fast and simple method of screening many genes. Because of the ease of knocking down gene expression, these large scale screens can be performed in high-throughput [163]. Additionally, screening protocols by pooling shRNAs in pools of up to up to 100 genes, allow us to study many genes thought to be involved in cancer biology and therapeutics [159]. Additionally, shRNA technology can be used to mimic suppression of transcription expression by methylation [152].

In order to identify which genes can affect the resistance to platinum drugs in ovarian cancer our lab has used several different assays. We hypothesized that the tumors will have taken advantage of epigenetic mechanisms to become resistant to therapy and/or increase their ability to survive and therefore examined DNA methylation

data for 50 ovarian tumor samples. To pull out gene candidates that are involved in therapy response for functional analysis we utilized data integration in a step-wise manner on a gene-by-gene basis examining whether these genes separate sensitive and resistant patients according to the clinical parameter (**Figure 2-1**). Therefore, candidate genes were first selected based on the ability to segregate resistant from sensitive tumors (**Figure 2-2**). Then, a t-test was performed for the methylation data to identify genes methylated that can segregate sensitive from resistant patients. The methylation data was next associated to identify which genes are repressed when methylated. This generated a list of over one thousand genes. The first aim of this dissertation was to generate a list of the most significant genes (about 350) and further validate them in the functional in-vitro screen according to **Figure 2-3**. These genes were compiled not only based on statistical significance, but also generated from pathway analysis and biological roles. For example, transcription factors such as Twist1 play an important role in gene regulation and have been hypothesized to play a role in chemoresistance and therefore it was included in the screen [164]. This list was then used for the shRNA pooling assay to pull out candidate genes that might have a functional role in therapy response.

In summary, the main goals on the functional validation arm of the study have been to: 1) perform pathway analysis to contextualize the results from statistical analysis (thousands of genes) 2) design the functional screen, 3) perform the functional screen 4) select a final candidate from the functional screen 5) functional validation of a gene from the functional screen (CHD3). The dissertation focuses on biomarker discovery for platinum resistance in EOC. The most significant genes (with the highest p-value) were pooled into RNAi assay to test for platinum sensitivity. The research question addressed

here aims at making sense of large amounts of genomic statistical data and translating it into meaningful biological candidates to understand a role for these genes in EOC chemoresistance. Furthermore, the data generated will have a global impact of the treatment of chemoresistant tumors.

2.2 Results

2.2.1 shRNA pooling

For each candidate gene, up to three miR30 shRNA clones in pLKO.1 and pGIPZ lentivirus vectors were selected from Open Biosystems Inc, propagated in bacteria and purified. Due to the large number of genes in the functional screen, we used a pooled shRNA approach. Each shRNA clone contains a GFP marker expressing from the same promoter as the shRNA hairpin and therefore we can continually monitor transfection efficiency after carboplatin treatment in both the control experiment and validation experiments. Moreover, sequencing of PCR-amplified shRNAs obtained from the cell lines indicate that our control gene was enriched during selection. Although pools of up to 100 clones have been found to be optimal for RNAi in other labs [159, 163], our results determined the optimal number of clones per pool was 50 (**Figure 2-4**). Thus, the purified plasmid DNA pools contained no more than 50 shRNA clones per pool. Screening of lower complexity pools is much more feasible and has been found to maximize the chances of identifying weaker shRNAs that might have been otherwise outcompeted in the pool [159]. Furthermore, no more than one shRNA per gene was

used in a single pool and there was one pool of GFP tagged pGIPZ empty vector to monitor transfection efficiencies. Efficiency of less than 70% was restarted (**Figure 2-4**).

DNA from the virus pools was transfected into 293T lentiviral packaging cell line (ATCC) following standard infection/transfection protocol. Briefly, 293T cells are transfected with lentiviral pooled shRNA vectors. Lentiviral supernatants are harvested 48-72 h post-transfection. Host cell lines are then infected with viral supernatant, and selected using carboplatin at the appropriate IC50 levels as previously determined (**Appendix 1**). The shRNA screens were carried out in 24 well plates due to the large number of assays (for work flow review, please see **Figure 2-3**)

2.2.2 Carboplatin screen

We presently have 14 ovarian cancer cell lines and have tested them for platinum resistance. Each cell line was cultured with increasing levels of platinum so that the final concentration in the media was from 1-100ug/ml. The IC20, IC50 and IC80 values were calculated for each of the cell lines (**Appendix 1**). From the 14 cell lines tested we originally chose HOSE 6-3, SKOV-3, CAOV3, and TOV21g for our functional studies. HOSE 6-3 is a normal human ovarian surface epithelial cell line that has been immortalized by HPV E6/7 ORF but the cells are not tumorgenic [165, 166]. This cell line has been used before in the study of chemoresistance and has been found to be moderately sensitive to platinum treatment (medium resistance) [165]. CAOV3 is an ovarian cancer cell line derived from epithelial tumor and shows a low sensitivity to platinum as determined by us and others. SKOV-3 was found by us to be the most

resistant to platinum and is so designated by ATCC. TOV21g is known to be the most sensitive to platinum, so much so that it did not work out well in the screen and we had to remove it from future pools. Survival curves for the cell lines used in the experiments can be seen in **Appendix 1**.

After the ovarian cell lines are transfected with the clones they are challenged with the appropriate IC50 value of carboplatin as previously determined. After treatment, DNA from the cell lines was harvested and the population of clones was amplified using universal primers by polymerase chain reaction (PCR). After amplification, the DNA was run on a gel and the band correlating to the shRNA insert size was cut out from the gel and DNA was extracted using the Qiagen Gel Extraction Kit. Finally, the DNA was transformed and cloned into a sequencing vector. These clones are sent for sequencing and positive hits are individually verified using BLAST analysis.

2.2.3 shRNA screen successfully identifies candidate genes

Altogether we have designed 7 pools of about 50 genes/pool in triplicate for each of the above mentioned cell lines. My pooled results indicated several hits, listed in **Table 2-1**. These positive hits underwent single clone validation using a similar screen as mentioned above and seen in **Figure 2-3**. Briefly, shRNAs were transfected and infected into the ovarian cancer cell lines (Hose 6-3, CAOV3 and SKOV3). Next, after infection was confirmed to be about 70-80% using GFP reporter (**Figure 2-4**), cells were subjected to varying doses of carboplatin corresponding to their IC20, IC50 and IC80 values. Unlike the pooled experiments, carboplatin resistance was measured over a 3

cycle period, being subjected to carboplatin for 72 hour on and 72 hours off, for a total of 18 days. This was done to more closely mimic the chemotherapy treatment patients receive in a clinic. After the 3 cycle period, results were analyzed and survival was calculated using the MTT assay. Each hit was tested in-vitro in triplicate 96-well plates. Positive hits were identified as being able to survive up to their IC50 values in all 3 ovarian cell lines and are listed in **Table 2-1.** Carboplatin survival data as well as RT-Q-PCR validation on these clones can be seen in **Appendix 2-3** and **Appendix 3.**

The sequence of events that lead to platinum resistance is complicated. The in-vitro screen identified several candidate genes that may play a role this chemoresistance. One candidate, a chromodomain helicase DNA binding protein 3 (*CHD3*) was further characterized for its functional role in resistance. Two independent shRNAs for *CHD3* were isolated per in vitro screen in two separate vectors, pLKO and pGIPZ. Furthermore, we hypothesized that because chromodomain helicase DNA binding proteins play an important role in chromatin regulation, it may serve an important function in chemoresistance. Understanding the complexity of the combinatorial arrangement of chromatin remodelers appears to be a challenge for many biologists [12]. Therefore, understanding a role for CHD3 in not only chemoresistance but gene expression patterns is important not only for cancer research, but evolution and development as well [122, 167]. Pointers from the literature revealed that not much is known about this protein and therefore it was intriguing to follow up on. Additionally, recent research demonstrated that Chd5, a subfamily of chromatin remodelers sharing structural and sequence conservation to Chd3, is frequently deleted in human neuroblastomas [129].

Because the shRNAs have not been tested for efficient functional repression of their target genes we next checked transcriptional repression using both RT-Q-PCR and Western blotting. DNA from the cell lines was harvested and the population of clones was amplified using universal primers by polymerase chain reaction (PCR). As seen in **Figure 2-5, Panel A-B** efficient knock down of *CHD3* in vitro at both the mRNA level (**A**) and translation of the protein was inhibited (**B**), as compared to the controls. Additionally, a second shRNA was also used to rule out an off target effect and the subsequent RT-Q-PCR results for the pLKO vector clone can be seen in **Appendix 2-4, Panel A**.

2.3 Discussion

The observations described here have enabled one of the first large-scale classifications of epigenetically controlled genes on the basis of their role in therapy response. Furthermore, this study also identifies a large group of genes whose loss of function results in marked chemoresistance, and therefore may indicate a previously unrecognized role for these proteins. Finally, our data demonstrate that downregulation of a novel chromatin remodeler causes resistance in EOC to high concentrations of platinum agents. Therefore, we hypothesize that chromatin remodelers are potentially important biomarkers for ovarian cancer.

Development of in vitro experimental models that accurately recapitulate genetic events that occur during human epithelial ovarian cancer (EOC) initiation and progression is crucial for a better understanding of EOC pathogenesis, identification of

early disease markers, and development of more effective therapy. The results from the functional validation screen have allowed us to take critical steps into understanding the mechanism that promoter methylation plays in therapy response of ovarian cancer. Specifically, to identify the genes involved in therapy response, we performed an oncogenomics-directed RNAi screen for shRNAs that promote survival of ovarian cancer cells in-vitro (see **Figure 2-2 and Figure 2-3**).

Epigenetic inactivation of genes is crucial for control of normal cell growth and is a hallmark of cancer [1]. Epigenetic silencing occurring during tumor development has the potential to affect the chemo-sensitivity of tumor cells and how tumors respond to chemotherapy [109]. We have looked at the epigenome of ovarian cancer to study genes suppressed by DNA methylation and demonstrate that there may be several genes that play an important role in chemosensitivity.

A great amount of information can be obtained from performing large scale bioinformatic screens. But, only a small percent of the genes in the informatics screen passed the RNAi functional validation indicating that it is likely there were a number of false positives. This could be due to several reasons. First, there is some technology limitations since we are averaging, there is some biological variability because in many studies only a few samples are affected (e.g. only 30% of samples have p53 mutation). So, we are limited to the samples that are provided, which are often heterogeneous. This would also be the case for the search for a biomarker, in that it may only hold true for a limited number of samples due to biological variability of the samples. Furthermore, the shRNAs from Open Biosystems have not been functionally tested for repression of their target gene and therefore we may have several weak shRNAs from the screen. Therefore

the design of a better library is essential for these genome wide RNAi screens. Another possibility of the shRNA not working is that the targeted gene may have low expression in the cell lines used, which is why we used three cells lines for this screen, although it is still a potential possibility. Therefore, other genes are likely to have a role in chemoresistance but we were just unable to pull it out in this screen. It would be beneficial to add additional cell lines and/or check expression analysis for the gene hits in the cell lines used. Moreover, in vitro tissue culture models are used for rapid screening of large numbers of genes; however the shRNAs may attribute selective dependency to culture conditions. Therefore, it will be essential to test the functional validation of the top hits from the screen in vivo for further confirmation of the role the gene(s) play in therapy response. Additionally, the bioinformatics are continually evolving and depending on how you perform the informatics, the list may fluctuate. The microarray data we used was analyzed using a layered approach with integration of the data and association with therapy response in a step wise manner. Some samples may have a low correlation with other samples and some probes on the microarray may not report well due to low tumor content of the sample. We are continually developing our informatics to get better correlation between tumor samples and therapy response. As expected there will always be some level of false positives in the informatics screen. This work is part of the ongoing project in the lab.

The therapy response genes characterized here target a remarkable array of biological activities, as listed in **Table 2-2**. There are several interesting genes that came out of the screen and further work in the lab will focus on their characterization. This

dissertation will just focus on one candidate, *CHD3*, for the reasons outlined in the previous section (chromatin remodeling, gene expression regulation, etc).

Oncogenic activation of the Wnt signaling pathway is common in cancers. Interestingly, as noted from our gene hit list, we had several genes validate from the Wnt pathway. Recent reports demonstrate that the overactivation of the Wnt pathway may be an important player in drug resistance, particularly the role of GSK3β [168]. Furthermore, the Wnt pathway has recently been describe as a possible player in chemoresistance in a number of tumors including hepatocellular carcinoma [169], neuroblastoma [170], and colorectal carcinomas [171]. Specifically, in ovarian cancer, the Wnt pathway has a potential role as a prognostic marker and therapeutic target [172, 173]. Additionally, Su et al. (2010) report that epigenetic silencing of SFRP5 leads to oncogenic activation of the Wnt pathway and contribute to ovarian cancer progression and chemoresistance through the TWIST-mediated EMT and AKT2 signaling [172]. These recent results highlight the importance of our in vitro functional screen as well as serve as validation for our gene hits (**Table 2-1** and **2-2**)

This study provides a genomic view on the role of epigenetics in the regulation of therapy response. It is increasingly important to understand the effects of treatment drugs on cellular pathways and networks. Microarrays provide a novel way to investigate the expression of thousands of genes simultaneously, with a view to dissecting the molecular pathways [174]. The preliminary pathway analysis has given us some clues into how DNA methylation may contribute to the disruption of key biological pathways during ovarian tumor progression to a drug-resistant phenotype. A number of statistical tests combined with known biological databases for detecting not only significant pathways

but principle components of these pathways. One of these databases was DAVID (**D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery) [175, 176]. We have identified enriched biological themes and discovered functional-related gene groups using the 350 candidate genes. We conducted a detailed analysis of tumor sample and patient survival data. By examining the data as separate analyses sets: tumors vs. normal and resistant vs. sensitive, we have been able to cluster our gene set and determine commonalities in biological processes and molecular functions. Tops genes included pathways in cancer (**Table 2-2**). Although the clustering pathway analysis from all our candidate genes revealed interesting information, it is more interesting to see our single clones cluster into certain biological processes. Pathway analysis can be further validated using the respective assays for the pathway involved. We hypothesize that aberrant DNA methylation may contribute to the disruption of key biological pathways during ovarian tumor progression to a drug-resistant phenotype [108, 177-179]. Again, this highlights that we can use statistically significant genes found in the bioinformatics screen to identify biologically important genes and pathways that can be used to treat cancer patients. This will be the focus of future work in the lab.

2.4 Methods

Materials

HOSE 6-3, a normal human ovarian surface epithelial cell line that has been immortalized by HPV E6/7 ORF. CAOV-3- (ATTC# HTB-75) and SKOV-3 (ATTC#

HTB-770), ovarian adenocarcinoma cell lines, were obtained from ATCC (Manassas, VA), along with the 293T packaging cell line (ATTC# CRL-11268). All cell lines were maintained in DMEM media with 2 mM L-glutamine, 4.5 g/mL glucose, 50 U/ml penicillin, 50 mg/ml streptomycin, and 10% fetal bovine serum, at 37°C and 5% CO_2. Cell culture reagents and the Virapower helper plasmid for lentiviral infection was obtained from Invitrogen, Carlsbad, CA. MiR-30-lenti-based shRNA clones in pGIPZ vector were obtained from Open Biosystems, as well as the Arrest-in transfection reagent. Polybrene was obtained from Millipore. SYBR Green I fluorescent dye, Reverse Transcriptase and RNase Inhibitor was purchased from Applied Biosystems, Foster City, CA. *CHD3*, β-Tubulin, and secondary antibody (horseradish peroxidase HRP-conjugated) were obtained from Cell Signaling. The Western Lightning ECL-plus detection system was obtained from Perkin Elmer. Transwell assays and reagents were obtained from BD Bioscience. Qiaquick gel extraction, RNEasy, Qiaquick MiniPrep, All Prep DNA/RNA, Minelute PCR Purification, DNEasy are all various kits used and were all obtained from Qiagen. Cisplatin, Carboplatin, and Staurosporin were purchased from Sigma. The radioactive version of Carboplatin was supplied by Accelerated Medical Diagnostics. MTT assay kit was supplied by Roche. BRDU assay kits were supplied by Calbiochem.

Chemotherapy Sensitivity

Ovarian cell lines HOSE 6-3, SKOV-3, and CAOV3 were tested for Carboplatin and cisplatin sensitivity (see **Appendix 1**). Each cell line was seeded at 10^3cells in a 96 well plate and treated with either chemotherapy drug at a concentration range of 1-100ug/ml in 100ul of fully supplemented DMEM. After a period of 72 hrs the drug was aspirated and proliferation was assessed by MTT assay. The IC20, IC50 and IC80 values were calculated for each of the cell lines using.

Chemotherapy Survival

To more accurately mimic clinical chemotherapy treatment in cell culture, cell lines were seeded at 10^3 cells per well in a 96 well plate and subjected to three cycles of a 72 hour treatment followed by a 72 hour recovery period for a total of 18 days. The cells during the treatment period were treated, in triplicate, at their respective IC20, IC50, IC80 concentrations with a triplicate set of no treatment controls. Following the final treatment cycle the cell proliferation was assessed by MTT assay. Dose responses were graphed using Microsoft Excel.

Introduction of short Hairpin RNA lenti viral vector

Lentiviral-mediated gene transfer was performed following the standard infection/transfection protocol (Open Biosystems). The 293T cells were transfected with lentiviral shRNA vector clones supplemented with the Virapower helper plasmid and the Arrest in transfection reagent. Experiments were performed in triplicate. Efficiency of the

transfection was determined using a GFP construct in the shRNA vector clones. Efficiency of transfection less than 80% was restarted. Lentiviral supernatants were harvested 48-72 h post transfection, filtered in a .45 micron syringe filter, supplemented with 4 µg/mL polybrene and used to infect the parental cell lines. Infected parental cells are selected for using puromycin at the minimal concentration needed to kill non-infected cells; this was determined by using non-infected parental controls and a period of at least 72hrs for selection. RNA was extracted from cell lines using the Qiagen RNEasy kit and treatment with the DNase RNase-free set (Qiagen, Valencia, CA, USA), all according to manufacturers' instructions. DNA/RNA was quantified using Nanodrop 1000 (Nanodrop); quality was assessed using the 2100 Bioanalyzer (Agilent Technologies, Canada). Efficiency of shRNA suppression of message was tested by Q-RT-PCR using the actin or GADPH gene as an internal control.

Immunoblotting

Samples were harvested from the different cell lines, total protein was extracted and protein concentration was determined using the Bradford Assay (Bio-Rad) as per manufacturer's instructions. Western blotting was performed using 5-10 µl of protein lysates at about 1µg/µl, according to standard procedures using the Invitrogen E-Page® system. Immunodetection was done using anti-rabbit monoclonal antibody against *CHD3* (Cell Signaling), diluted 1:1,000, followed by incubation with anti-rabbit secondary antibody (horseradish peroxidase HRP-conjugated) (Cell Signaling), diluted 1:10,000. Anti-rabbit monoclonal β-Tubulin was used as a positive control . The Western

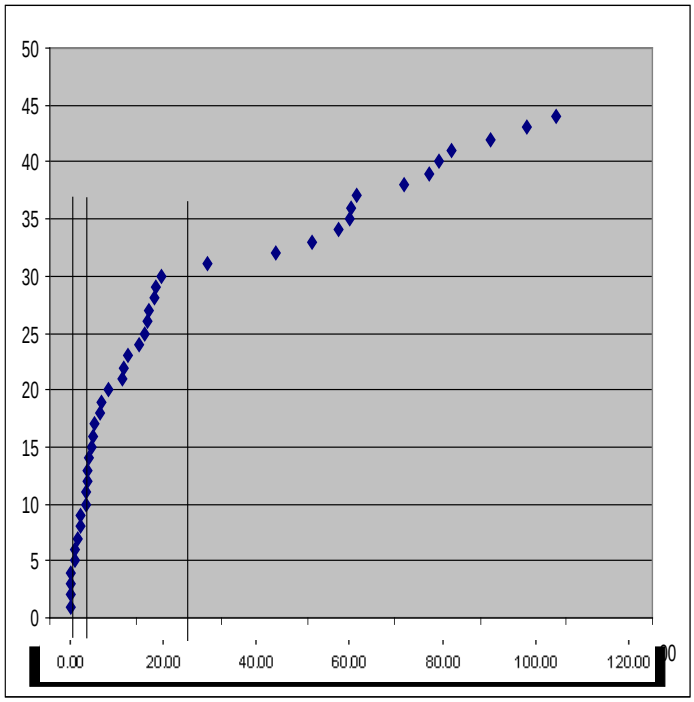
Lightning ECL-plus for HRP conjugated antibodies detection system (Perkin Elmer) was used for signal detection. Quantification of protein lysates was done using ImageJ, a public domain Java based image processing program, available for download at http://rsbweb.nih.gov/ij/

Gene Enrichment Analysis

The Database for Annotation, Visualization and Integrated Discovery Bioinformatics (DAVID) was used to annotate genes from our hit list. DAVID Bioinformatics Resources (DAVID) at http://david.abcc.ncifcrf.gov consists of an integrated biological knowledgebase and analytic tools aiming at systematically extracting biological meaning from large gene/protein lists. Enrichment analysis is a strategy that increases the likelihood for investigators to identify biological processes most pertinent to the biological phenomena under study.

2.5 Figures

Figure 2-1



- Refractory: (PFI<1m)
- Resistance : (1m< PFI<6m)
- Sensitive – early: (>6m PFI < 24m)
- Sensitive –(>24m PFI <120m)

Figure 2-1: This figure represents the ovarian cancer clinical sample categories. It demonstrates how we grouped primary tumor samples into several categories based on their PFI's or platinum-free intervals, sometimes also referred to as progression free intervals. This is defined as the time period after platinum treatment in which there is no observed progression of the disease. If the PFI<1m it is referred to as refractory, between 1m and 6m is considered resistant, between 6m-24m is early sensitive, and over 24m is sensitive. The y-axis lists the tumor sample number and the x-axis represents the progression time in months.

Figure 2-2

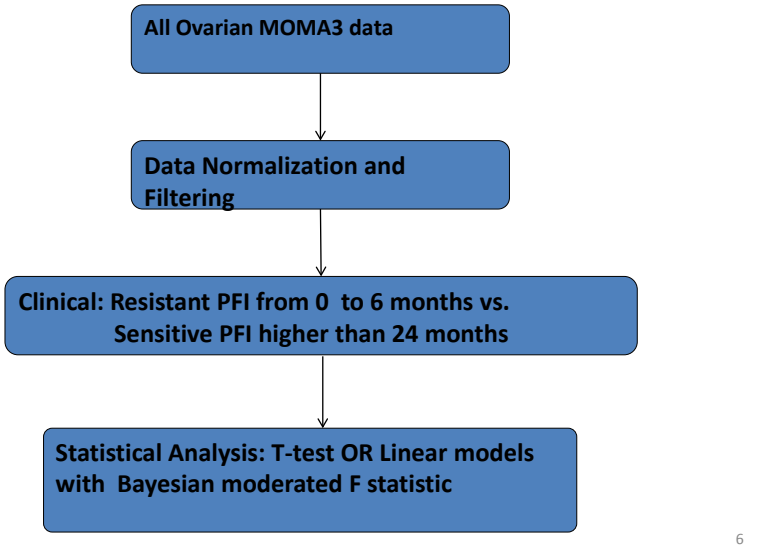


Figure 2-2: is the schematic for the overall flow of work for obtaining the list of genes for the pooling experiments. This approach for data analysis was a layered approach with the integration of the data and association with therapy response in a stepwise manner. Candidate genes are those that can separate the tumor based on therapy response.

Figure 2-3

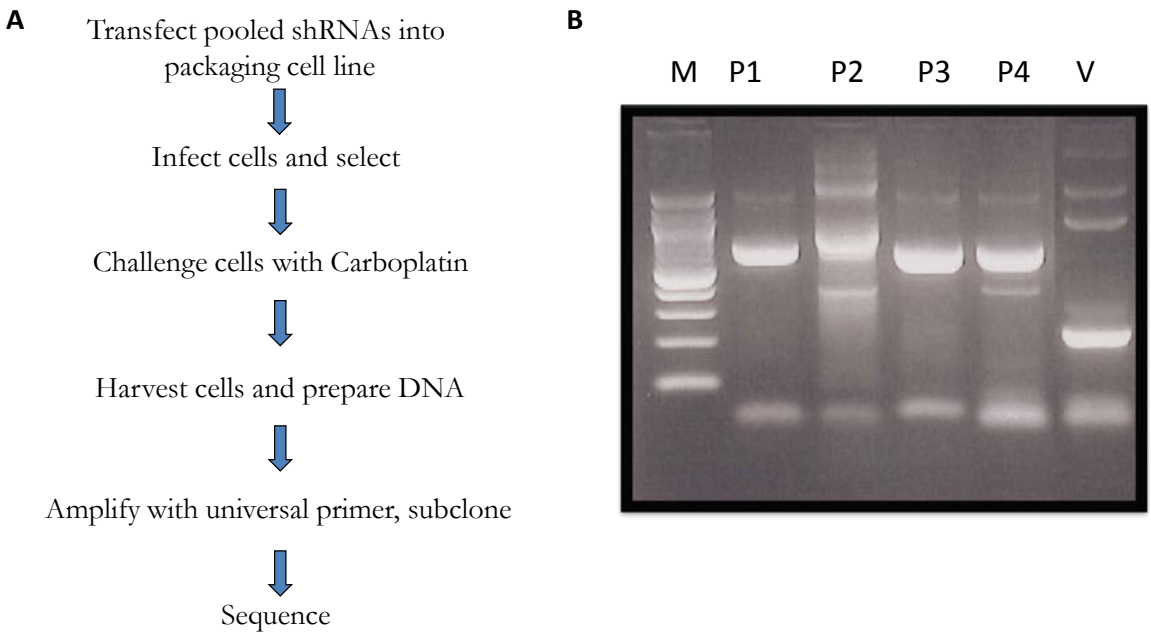


Figure 2-3: **Panel A** represents the schematic for the overall progression of the functional validation experiments for both single clones and pooled shRNAs. **Panel B** represents an ethidium bromide stained 1% agarose gel identifying single bands for several different pooled shRNA populations (P1-P4) and the vector only control (V).

Figure 2-4

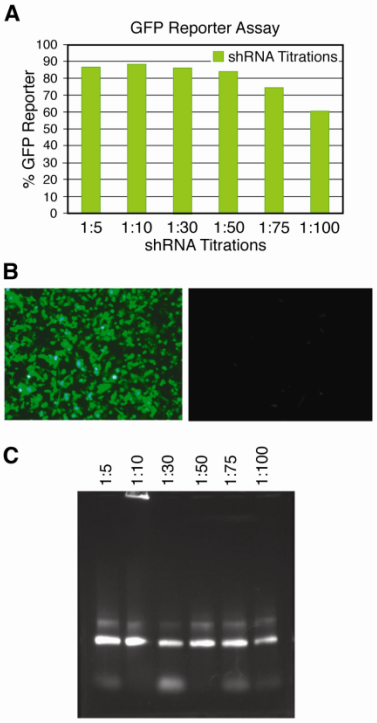


Figure 2-4: This figure represents the experimental controls for the functional assay using miR30 shRNA clones in a pGIPZ lentivirus vector from Open BioSystems. **Panel A** is a bar graph plot and illustrates the transfection efficiency of the pooled shRNA approach. Each shRNA clone contains a GFP marker expressing from the same promoter as the shRNA hairpin and can therefore be continually monitored in culture. The x-axis lists the dilution ratios of clones starting from pools of 5 and going up to pools of 100. The y-axis represents % of GFP reporter as seen using a phase-contrast microscope. For our experiments, the purified plasmid DNA pools contained no more than 50 shRNA clones per pool. Efficiency of less than 80% was restarted. **Panel B** is a representation of what the shRNA pools cells looked like under the microscope when they were selected for in vitro vs. the control population of cells with no shRNA. Magnification 20x. **Panel C** represents an ethidium bromide stained 1% agarose gel identifying single bands for the different ratios of the pooled population of shRNAs. Ratios are listed above the gel.

Figure 2-5

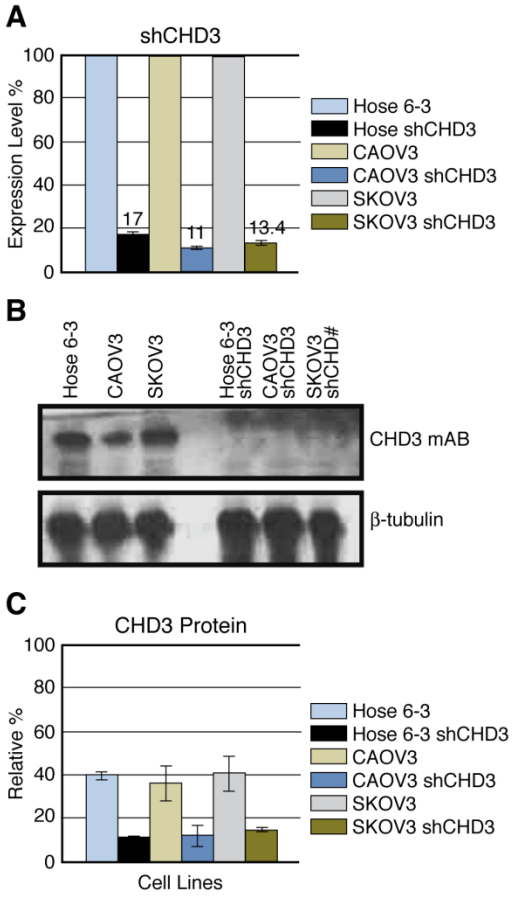


Figure 2-5 shows validation of specificity of the shRNA clones targeting *CHD3*. **Panel A** is a bar plot of RT-Q-PCR analysis of the shRNA infections calculating the efficiency of shRNA induced knockdown relative to the control non target shRNA infection. The Y axis is the percentage transcript with the non target shown as 100% by definition of being the reference. Each bar represents one of the infections in one of the three cell lines used. Inset on top of the bars for cell lines with *CHD3* shRNA is the level of transcript determined to be remaining after shRNA induced knockdown. RT-Q-PCR was done in triplicate and graph represents averages of three experiments; $P<0.04$. **Panel B** represents a western blot analysis. The top panel shows the western blot of shRNA infections demonstrating efficient knockdown of the protein. The bottom panel is the B-tubulin loading control. The blot represents efficient inhibition of protein translation. Above the blot are the names of the cell lines used in this experiment. Experimental translation knockdown of CHD3 was decreased to <10%. **Panel C** shows the quantification of specificity of shCHD3 clones targeting CHD3 at the protein level. Bar graphs represent quantification of protein knock down in the cell lines. Error bars represent the standard deviation ($n=3$, $p<0.04$). ImageJ was used for protein quantification.

Table 2-1

Gene	IC 20	IC 50	IC 80
BRCA2	+	-	-
CAMK1D	+	+	-
GSK3B	+	+	+
DOK2	+	+	+
COX5A	+	+	-
APRT	+	+	-
BMP2	+	+	+
ARF4	+	+	-
OXSR1	+	+	-
CENPB	+	+	-
FZD1	+	+	+
ESRRA	+	-	-
HIRIP3	+	+	-
GTF2b	+	+	-
SGPL1	+	+	-
GABPA	+	+	-
TWIST1	+	+	+
SNX2	+	-	-
MDH1	+	+	+
MRPL33	+	+	-
NR2E1	+	+	-
NR3C2	+	+	-
SOX9	+	+	+
VIPR2	+	-	-
TOB1	+	+	+
UNG	+	+	-
ZIC1	+	+	+

Table 2-1 Gene Hit List
This table represents the genes that scored from the pooled shRNA functional analysis. Each gene was underwent single clone analysis with a 3 cycle treatment of platinum drugs at the various Ic20, Ic50 and Ic80 concentrations. Results for each dose are listed here.

Table 2-2

Term	P-Value	Genes
DNA binding	0.0010	CENPB, GABPA, NR3C2, GTF2B, TWIST1
phosphoprotein	0.0039	SGPL1, ESRRA, UNG, SNX2, NR3C2, BRCA2, OXSR1, VIPR2, NR2E1, GTF2B, APRT, DOK2, GSK3B, CENPB, HIRIP3, TOB1, CAMK1D, MDH1
DNA-binding	0.0101	ESRRA, CENPB, GABPA, NR3C2, SOX9, ZIC1, NR2E1, TWIST1
nucleus	0.0176	ESRRA, UNG, CENPB, GABPA, NR3C2, HIRIP3, SOX9, ZIC1, GTF2B, NR2E1, TWIST1, CAMK1D
developmental protein	0.0196	BMP2, FZD1, ZIC1, NR2E1, TWIST1
transcription regulation	0.0493	ESRRA, GABPA, NR3C2, SOX9, GTF2B, NR2E1, TWIST1
Transcription	0.0540	ESRRA, GABPA, NR3C2, SOX9, GTF2B, NR2E1, TWIST1
disease mutation	0.0587	UNG, NR3C2, BRCA2, SOX9, APRT, TWIST1
transcription factor	0.0843	GABPA, TWIST1
serine/threonine-protein kinase	0.0931	GSK3B, OXSR1, CAMK1D

Pathway	*Count*	*P-Value*	*Genes*	*Fold Enrichment*
Basal Cell Carcinoma	3	0.005935233	BMP2, GSK3B, FZD1	23.11363636
Pathways in Cancer	4	0.029767721	BMP2, GSK3B, FZD1, BRCA2	5.167682927

Table 2-2 DAVID Analysis
Positive hits from the functional analysis screen were further evaluated using The Database for Annotation, Visualization and Integrated Discovery Bioinformatics (DAVID). As seen in the table, genes can easily be divided into certain pathways and biological processes.

Chapter III: Resistance to Platinum Causes Proliferation Changes associated with knockdown of *CHD3* In-Vitro

3.1 Introduction

Cellular proliferation of cancer cells is aberrantly regulated. Cancer cells generally contain the full complement of biomolecules that are necessary for survival, proliferation, differentiation, cell death, and expression of many cell-type-specific functions. Failure to regulate these functions properly, however, results in an altered phenotype and cancer. The rate of cell proliferation within any population of cells depends on three parameters: the rate of cell division, the fraction of cells within the population undergoing cell division, and the rate of cell loss from the population due to terminal differentiation or cell death [180]. Proliferative activity is known to be related to chemoresponse. Although increased cell proliferation is a hallmark of cancer cells, some reports hypothesize that low proliferation activity may be associated with chemoresistance. One report by Itamochi *et al*. (2002) examined cellular proliferation in clear cell carcinoma and concluded that lower proliferation of tumor cells may be a behavior of clear cell carcinoma of the ovary and that this contributes to its resistance to chemotherapy [181].

Chromatin remodeling is essential for many processes including transcription. The ATP-dependent chromatin-remodeling protein *CHD3* is part of the NuRD complex in mammalian cells which contain histone deacetylases (HDACs) and function as a transcriptional repressor [182]. Chromatin is dynamic, with its structure and its histone

modifications continually changing during transitions in development and in response to extracellular cues. Furthermore, ATP-dependent enzymes that remodel chromatin are important controllers of chromatin structure and assembly, and are major contributors to the dynamic nature of chromatin, particularly during development. Some recent reports suggest that these protein complexes are essential for establishing and maintaining pluripotent and multipotent states in cells [116].

Here, we report that when *CHD3* is silenced using a mIR based short hairpin RNA, cells in vitro display a unique slow growth phenotype. Furthermore, these cells are also resistant to platinum based therapy, carboplatin and cisplatin. Taken together, these results suggest that *CHD3* is epigenetically regulated gene that is transcriptionally repressed and plays an important role in platinum-based resistance in ovarian cancer cell lines and primary ovarian tumors.

3.2 Results

3.2.1 Characterization of sh*CHD3* cells

Affymetrix expression array data for a panel of ovarian cell lines was used to identify cell lines with moderate expression levels of the *CHD3* gene. The cells lines used for the screen were a normal ovarian epithelial cell line HOSE 6-3, immortalized by the introduction of the HPV E6/7 ORF, and two tumor cell lines, CAOV3 and SKOV3. The shRNA construct for *CHD3* was introduced into each of the cell lines and then

challenged with Carboplatin for three cycles of three days of Carboplatin treatment followed by three days off. In addition three concentrations of Carboplatin, IC20, IC50, and IC80 were used. Each of these lines with the shRNA to *CHD3* show increased survival of cells when challenged with Carboplatin (**Figure 3-1 Panel A**). In fact, CAOV3 sh*CHD3* cells have about an 85% survival rate and are up to four times more resistant to carboplatin than CAOV3 cells without the shRNA. These experiments were repeated using Cisplatin, a sister molecule to Carboplatin, and cells with the shRNA to *CHD3* showed a marked resistance to doses up to the IC80 value determined for Cisplatin (**Figure 3-1 Panel B**). The proliferation and carboplatin resistance experiments were repeated using a second shRNA in the pLKO vector and the results can be seen in **Appendix 2-4 Panel B and C.** We validated all the positive hits from our functional pooled screen using the 3day, 3 cycle carboplatin treatment. Results from those experiments can be seen in **Appendix 2-4**.

3.2.2 *CHD3* proliferation analysis

Interestingly, the ovarian cancer cell lines in vitro that were silenced for *CHD3* appeared to grow slower. Therefore, it was important to test if these cells were dying due to platinum treatment or just not proliferating as fast as their controls. As seen in **Figure 3-2, Panel A** cells with the shRNAmir to *CHD3*, are viable over a 3 cycle 18 day period of platinum treatment. Strikingly, these results suggest that the silencing of *CHD3* is indicative of a more resistant phenotype to platinum therapy. To establish that just the growth of the cells was being inhibited and that the cells were still viable and not growing

and then dying, we performed a BrdU assay. Measurement of BrdU, a thymidine analog, is incorporated into newly synthesized strands of DNA in actively proliferating cells. Because *CHD3* is a chromodomain helicase DNA binding protein and part of the NURD complex it is presumed to be involved in the remodeling of chromatin by deacetylating histones and therefore may play an important role regulating genes involved in cell proliferation. As noted, we found that when the small hairpin RNA to *CHD3* is introduced into cells and challenged with platinum, the cells are able to escape and survive the treatment better than their controls. As seen in **Figure 3-2 Panel B**, cells both with and without the shRNA to *CHD3* are actively proliferating, however the sh*CHD3* cells are proliferating at a slower rate. These results indicate that there is a decreased number of dividing cells, a slower rate of cell division or, thirdly, a combination of both in the population of cells silenced for *CHD3*.

In conclusion, suppression of *CHD3* in all three ovarian cell lines demonstrates that proliferation was slower in these cells than their respective parental cells as determined by the MTT and BrdU assays. However, viability was not necessarily affected. Therefore, cells with the shRNA to *CHD3* are able to escape treatment with platinum drugs by proliferating at a slower rate. It will be interesting to see if this is the case for other non-platinum based chemotherapeutic drugs, such as taxol.

3.2.3 *CHD3* cellular location and function

Members of the *CHD3* subfamily are characterized by the presence of two tandemly arranged chromodomains as well as the presence of one or two PHD (plant-

homeo-domain) zinc fingers preceding the chromodomains [117]. In animals, *CHD3*-class proteins exist in diverse multiprotein complexes including the Mi-2/NuRD (nucleosome remodeling and histone deacetylation) complex, which involve histone deacetylase and the dMEC complex, which represses target gene expression in a histone deacetylation-independent manner, although some *CHD3*-class proteins exist as a monomer [182]. This suggests that the *CHD3* protein localizes in the nuclear region. As seen in **Figure 3-3**, the nuclear localization was confirmed by immunofluorescence staining showing that *CHD3* is localized to the nucleus. During mitosis, the nuclear envelope breaks down and confocal immunofluorescence results show that *CHD3* protein is dispersed throughout the nuclear region (**Figure 3-3, panel B**).

ChIP-on-chip is a technique that combines chromatin immunopercipitation with microarray technology. It can be used to investigate the interaction of DNA and proteins, specifically those operating in the context of chromatin. As seen in **Figure 3-4**, the chip-chip analysis reveal that *CHD3* binds to several gene promoters. More specific, *CHD3* binds to the *FOXD3* promoter. We hypothesize that *CHD3* plays a dynamic role in chromatin remodeling. Furthermore, chromatin remodeling is required for transcriptional regulation and therefore we hypothesize that *CHD3* plays an important role in both transcriptional activation and repression.

3.3 Discussion

As drug resistance is one of the major barriers to the successful treatment of malignancies, investigation of the mechanisms of drug resistance and approaches to

overcoming it has been widely performed in the past decades. Several mechanisms have been proposed that enable cancer cells to avoid damage by chemotherapeutic agents. These include altered drug transport, drug inactivation, altered drug target, drug sequestration, increased DNA repair, and increased DNA damage tolerance. Specifically, the latter part of the group which may be characterized by alterations in the cell cycle pathway thereby slowing down growth rate has been proposed as a way to avoid damage by chemotherapeutic agents. Recent work done in colon cancer suggests that this may be a common chemoresistance mechanisms [183]. Nam *et al*. found that colon cancer cells had a decreased S phase and increased cell cycle arrest and suggested cells that slowly proliferate are generally less sensitive to cytotoxic agents, and this may be a p53 dependent event. p53 accumulation plays a key role in EOC and various mutations of p53 are frequently detected in cancer cells, by altering cell cycle checkpoints [184, 185] .

Platinum based drugs are an antineoplastic drug in the class of alkylating agents, thereby stopping tumor growth by cross-linking guanine bases in DNA double-helix strands and directly attacking DNA. As this is necessary in DNA replication, the cells can no longer divide [186]. Therefore, we hypothesized that our slow growth phenotype in-vitro might be a predictor of resistance in the cell. When the cells are challenged with platinum based drugs, they are able to escape and survive the treatment better than the control cells. Even when challenged with high doses of the platinum based drugs, these cell lines still survive and proliferate in vitro.

Additionally, a decrease in the rate of cell proliferation might be a direct cause from nutrient deprivation which in turn induces cell cycle arrest. Therefore increasing distance from tumor blood vessels might decrease the rate of cellular proliferation [187].

Most chemotherapeutic drugs, including, possibly, biologic agents that target cell proliferation, are more effective against proliferating than quiescent cells [188]. Consequently, cells proliferating slowly due to increasing distances from tumor blood vessels are likely to be resistant to therapy. This might be an additional mechanism to explain the slower rate of cellular proliferation in the chemoresistant EOC cells. It would be interesting to determine CHD3 levels in the different regions of the tumor.

The other possibility for the slow growth phenotype is that chemoresistance is caused by suppressing and/or activating some key proliferation related genes as *CHD3* is a chromatin remodeler. Because our data confirmed that *CHD3* is a nuclear protein, it may act as a key regulator of several genes. Furthermore, chromatin remodeling is essential for many processes including transcription. *CHD3* protein is part of the PICKLE (PKL) and PICKLE RELATED2 (PKR2) in plants and is required for the expression of many genes that are repressed by Polycomb Group (PcG) proteins [11]. Recent results show that *CHD3* has trxG-like functions in plants and are required for the expression of many genes that are repressed by PcG proteins [11]. The exact role of PcG proteins in tumorigenesis is still unclear, but they are known to play a role in chromatin remodeling. Importantly, PcG proteins play a major role in cell proliferation, senescence and tumorigenesis [189]. Moreover, since *CHD3* is a nuclear protein, it has the ability to regulate genes involved in the cell cycle as well as proliferation. As the chIP-chip analysis reveals, one such gene *CHD3* is thought to regulate is the *FOXD3* gene, by binding to its promoter. Interestingly, the forkhead box transcription factor *FOXD3* is a stemness factor and is thought to be negative cell cycle regulators [190]. More data is

needed to confirm the role of *CHD3* on *FOXD3* and is part of ongoing analysis in the lab. Furthermore, we are still awaiting expression data analysis from the sh*CHD3* cell lines.

In conclusion, we hypothesize that *CHD3* plays a direct role in cell proliferation in a two dimensional cell culture model, thereby allowing a cell to escape damage caused by chemotherapeutic drugs. More work is needed to examine the specific role that *CHD3* plays in cell cycle regulation as well as determine what genes are activated and repressed by the chromatin remodeler. Additionally, because cell growth and proliferation was measured in an in vitro two dimensional assay, it is important to note that this phenotype may only be specific to this culture system. This growth may be artificial and dependent on the cell culture conditions.

3.4 Methods

Cell proliferation/viability assays.

Control or shRNA *CHD3* cells (1×10^4) were cultured in 96-well plates in triplicate. BrdU (BrdU proliferation kit, Calbiochem, LaJolle, CA), in fresh medium for 24h. Incorporation of BrdU into cells cultured in plates is calculated and quantified measuring absorbance at 450-595 nm using the Wallac spectrophotometer. Additionally, cells cultured in 96-well plates in triplicate were measured for proliferation/viability by the MTT assay as described [7]. Briefly, following treatment of the cells, the medium was replaced with fresh medium containing 1 mg/ml MTT and incubated for 4 h at 37°C. One volume of 20% SDS, 50% *N*,*N*-dimethylformamide was added and incubated

overnight at 37°C. Absorbance at 570 nm were determined in a 96-well spectrophotometer and means of the triplicates were calculated. Following no cellular background subtraction, all data were normalized to the MTT conversion activity of solvent-treated control cells. For DNA laddering, no confluent cells from one 150 cm^2 Petri dish were collected and the genomic DNA was isolated and analyzed as described previously [191].

Chemotherapy Sensitivity Survival

For single clone validation studies and a more accurate representation of carboplatin chemotherapy in cell culture, cell lines were subject to a 72 hour treatment IC50 dose of carboplatin followed by 72 hour recovery for a total of 12 days. Following the drug treatment, viable cell numbers were determined using the MTT assay as described above. Dose responses were graphed using Microsoft Excel. Experiments were performed in triplicate.

ChIP-chip

Confluent ovarian cell lines were trypsinized, pooled, and plated in growth medium on 10cm plates. When cells had reached 90% confluency, the cells were washed with PBS and harvested using a cell scraper. Cells were spun down in a 15ml conical at 1000xg for 3min. Cell pellets were resuspended in 2ml lysis buffer (150mM NaCl, 10% glycerol, 0.3% TritonX100, 50mM Tris 8.0, 0.5% protease inhibitor solution (Sigma)) and the cell pellet was disrupted by continual resuspension through an 18 gauge needle. The samples were centrifuged for 30min at 12,000xg to

pellet cell debris, and the soluble chromatin was harvested. The Bradford method was performed to determine the quantity of protein in the chromatin sample (Biorad). Immunoprecipitations were prepared with ~1mg chromatin and 2μg of hCHD3 antibody (Abcam). 10ng of purified DNA from ChIP samples were amplified by the non-biased Whole Genome Amplification Kit per manufacturer instructions (Qiagen). Reactions were purified using Qiaquick columns (Qiagen), and concentrated by centrifugation under vacuum. Samples were hybridized as previously described [192]

Immunofluorescence

1x10^6 cells/mL harboring sh*CHD3* and control cells were washed twice with PBS and fixed with 4%paraformaldehyde. Cells were stained with primary antibodies against human *CHD3* (Abcam) for 12 hrs at 4°C. Nuclei were then stained with 4′,6-diamidino-2-phenylindole (DAPI; 1 μg/ml) for 5 min. Fluorescence images were obtained using a confocal laser scanning microscope.

DNA/RNA Isolation

Total DNA or RNA was extracted from cell lines using the Qiagen DNEasy or RNEasy kit and treatment with the DNase RNase-free set (Qiagen, Valencia, CA, USA), all according to manufacturers' instructions. DNA/RNA was quantified using Nanodrop 1000 (Nanodrop); quality was assessed using the 2100 Bioanalyzer (Agilent Technologies, Canada). The DNA/RNA samples used were all of sufficient quality for further analysis.

3.5 Figures

Figure 3-1

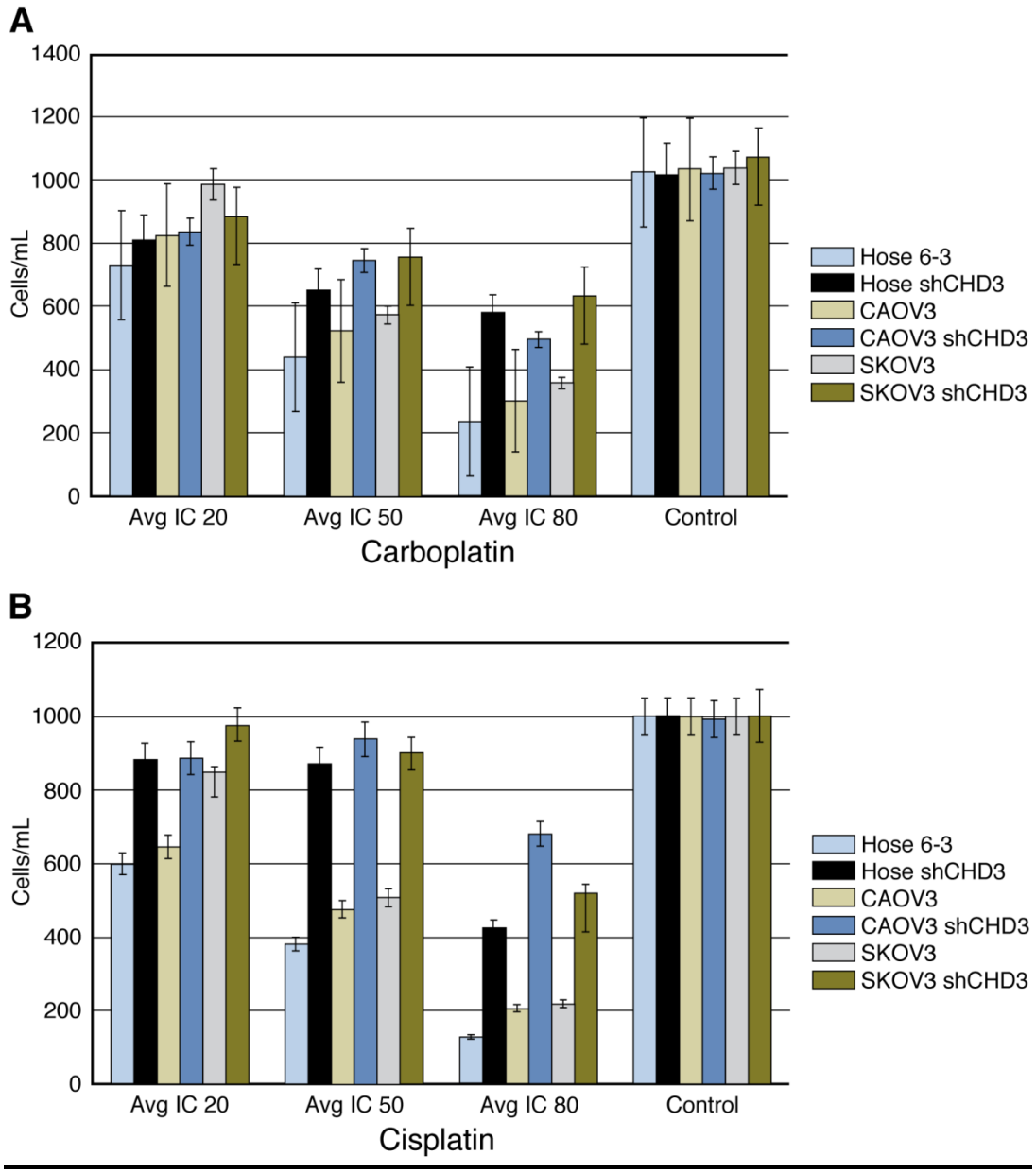


Figure 3-1: Downreulgation of CHD3 promotes resistance to platinum drugs. **Panel A-B** are bar graphs that represents cell survival over a 3 cycle, 18 day treatment of carboplatin (**A**) and cisplatin (**B**) drugs at the varying doses of IC20, IC50, and IC80. Cells were plated at 1x10^3 cells/ml in 96-well plates and treated with a 3-day cycle of platinum

drugs. Cell survival was measured after 18 days. Y-axis represents cell counts in cells/ml and the x-axis represents chemotherapy dose from IC20-IC80. Cell lines are listed on the left side of the panel. Each experiment was done in triplicate 96-well format. The bars shown are the means ± SD ($n = 12$; $P < 0.05$).

Figure 3-2

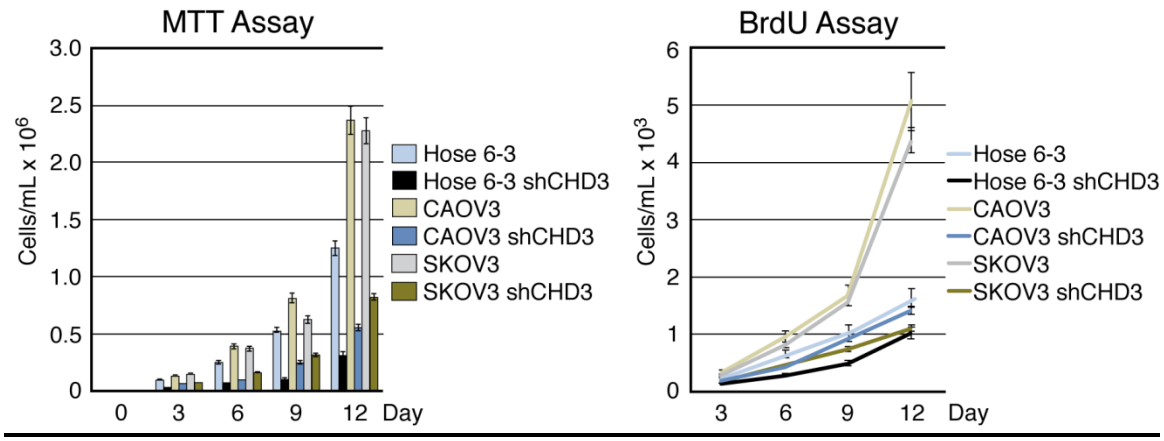


Figure 3-2: shows that *CHD3* shRNAs Cause a Slow Growth Phenotype. Cells were plated at 1x10^3 cells/ml in 96-well plates and cell growth was measured over 12 days. **Panel A** is a bar plot of growth analysis of the shRNA infections over a 12 day period. The x-axis represents the time line and the y-axis represents the cells/ml. This graph demonstrates that the shRNA to *CHD3* induced a slow growth phenotype relative to the control over the 12 day period. Cell lines are listed on the left side of the panel. The percentage of viable cells was calculated relative to untreated control. Each experiment was done in triplicate 96-well format. The bars shown are the mean ± SD (n = 12; P< 0.05). **Panel B** is a line graph of the Bromodeoxyuridine (BrdU) assay used to determine cell proliferation by incorporation of BrdU into the DNA of dividing cells over a 12 day period. The x-axis represents the time line and the y-axis represents the cells/ml. Each experiment was done in duplicate in 96-well format. Graph represents average from two experiments. Cell lines are listed on the left side of the panel. The error bars shown are the mean ± SD (n = 3; P< 0.06)

Figure 3-3

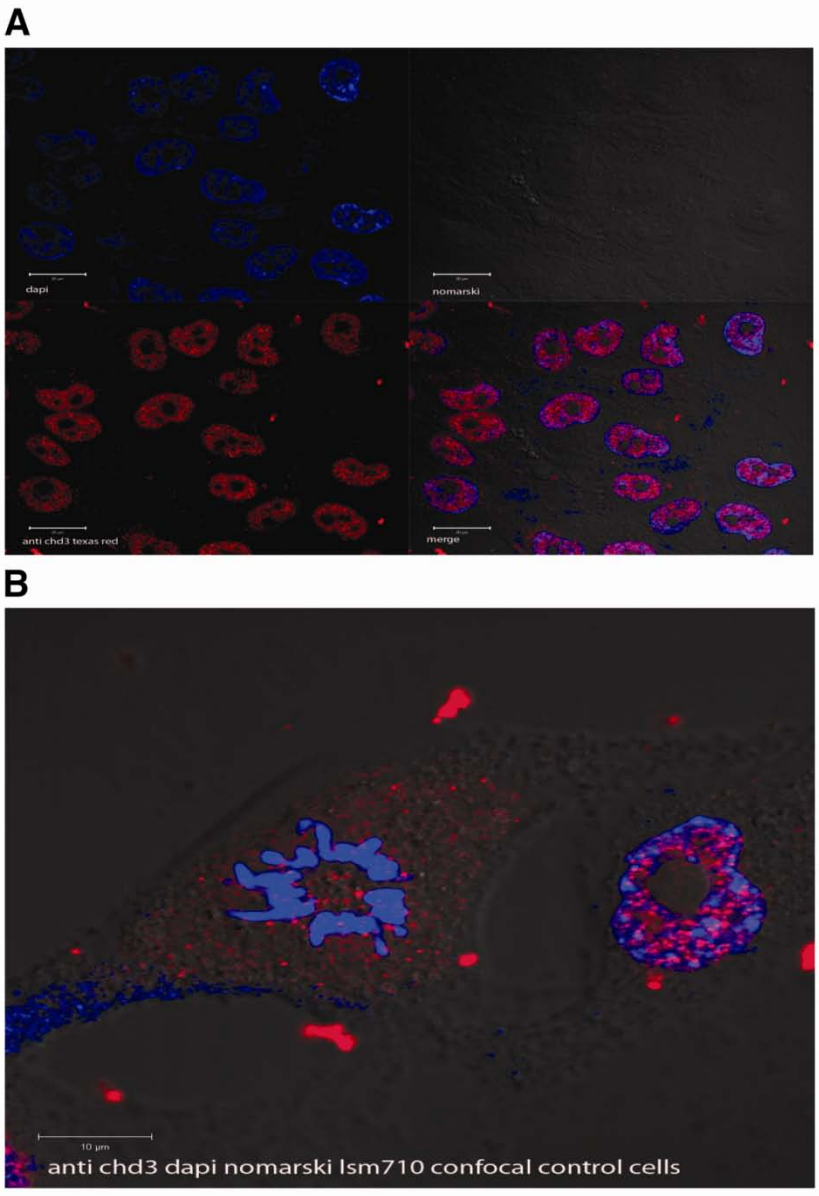


Figure 3-3: The Expression and Localization of *CHD3*. CHD3 is a nuclear protein involved in chromatin remodeling. **Panel A** Immunofluorescence staining shows that *CHD3* associates with DNA. Merge illustrates cellular association of *CHD3* with DNA. **Panel B** Confocal immunofluorescence to show *CHD3* protein in the nuclear region. Blue Dali represents DNA and Texas-Red represents the *CHD3* protein.

Figure 3-4

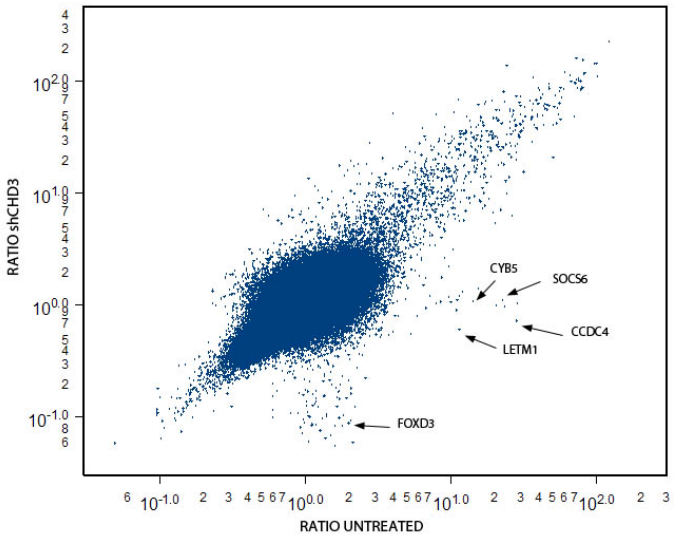


Figure 3-4 shows the result of ChIP chip assay with *CHD3***.** Antibodies to *CHD3* were used for chromatin immunoprecipitation, amplification of the DNA isolated from the IP and hybridization to a promoter array. Results of the array analysis are shown for a comparison of cells with a non target shRNA compared to cells with shRNA to *CHD3*. A number of promoter fragments were enriched in the non-target cells that were absent in the *CHD3* shRNA cells, including FOXD3, which is required for maintenance of pluripotent cells.

Chapter IV: Effect of *CHD3* Suppression on Uptake/Efflux [14]C Carboplatin

4.1 Introduction

The acquired mechanisms for resistance to platinum have been postulated to be multifactorial and illustrate the ingenuity of cancer cells to devising various maneuvers for survival of chemotherapeutic drugs. As listed in **Table 4-1** some of the mechanisms involve loss of the ability to import drug molecules through the plasma membrane or an acquired ability to pump drug molecules out through this membrane. These behaviors represent a challenge to all platinum based chemotherapy. One way decreased intracellular drug accumulation may involve the overexpression of P-glycoprotein multidrug efflux pump. This resistance is hypothesized to be different from the expression of the multidrug transporter encoded by the *MDR1* gene that protects cells from cytotoxic natural products [193] or by the *MRP1* gene that can efflux anionic drug conjugates [194, 195].

Reduced accumulation of cisplatin and carboplatin in resistant cells has been reported by several laboratories [196-198]. Christen *et al*. (1994) reported that both uptake and DNA intrastrand adduct formation were reduced in cisplatin-resistant cells using a tritium- labeled cisplatin analogue dichloro-(ethylenediamine)- platinum (II), whereas others have suggested that active efflux may exist in cisplatin-resistant cells [199, 200]. Additionally, one study reported that cross-resistance to a number of arsenic drugs were the result of an association with decreased expression of folate-binding protein and arsenic-binding proteins in cisplatin-resistant cells [201]. These results

suggest that selection for cisplatin resistance is a pleiotropic defect in uptake of several different unrelated compounds. We used C^{14} carboplatin to determine whether decreased accumulation of carboplatin in resistant cells was due to reduced influx or active efflux.

4.2 Results

There are three basic mechanism how cells can become resistant to platinum based chemotherapy. They can either develop a mechanism that allows decreased uptake of the chemotherapy, or a mechanism that allows them to remove the chemotherapy faster, or even allow the cells to tolerate the chemotherapy more. Of course some combination of these is also possible. Demonstrating that the cells with suppression of *CHD3* were more resistant to Carboplatin and Cisplatin, we next wanted to address if the resistance was a result of decreased levels of platinum in the cell. To do so we utilized C^{14} labeled Carboplatin and measured the influx. Cells were labeled with C^{14} Carboplatin and incubated for several time points from 0 to 48 hours (**Table 4-2**). We found that for all cells the influx or uptake of C^{14} Carboplatin was roughly the same with or without the shRNA for the *CHD3* gene. This demonstrates that the loss of *CHD3* does not affect the uptake of carboplatin into the cell.

We next tested the effect of loss of *CHD3* on the efflux or the cells ability to remove carboplatin. From the above experiment we observed that the inflection point for the influx of C^{14} Carboplatin was roughly 24 hours. Therefore to measure the efflux of C^{14} Carboplatin from the cells we first incubated the cells for 24 hours with C^{14} Carboplatin. Counting this point as time 0 we then washed the cells and collected cells at

several time points from 0 to 48 hours (**Table 4-3**). From the data we can see that there is no change in the ability of the cells to remove Carboplatin with or without suppression of *CHD3*. Therefore we have concluded that the cells have developed a mechanism to better tolerate the presence of Carboplatin.

4.3 Discussion

Chemotherapy with platinum based drugs is the standard treatment for ovarian cancer patients. Cisplatin and carboplatin form DNA interstrand and intrastrand crosslinks, and thus cause DNA damage and result in apoptosis of cells [202]. Mechanism of platinum resistance identified so far include alterations in cellular drug transport, enhanced DNA repair, and enhanced intracellular detoxification involving glutathione and metallothionein proteins [203]. Functional gene mutations or other changes that affect the expression of genes encoding proteins may influence drug resistance, such as epigenetic regulation [204].

Multidrug therapy strategies are often foiled by cancer cells, which develop powerful strategies for evading death, such as the acquisition of multidrug resistance (MDR). For example, high level expression of the MDR gene, which encodes a transmembrane drug efflux pump, enables cancer cells to efficiently excrete a variety of chemically unrelated drugs, thereby lowering the intracellular drug concentrations to subtoxic levels [205].

The limitations of clinical chemotherapy have been ascribed primarily to mechanisms that mediate drug resistance at the cellular level and the tumor

microenvironment. Moreover, the distribution of many drugs within tumors is heterogeneous, such that only a proportion of the target tumor cells are exposed to a potentially lethal concentration of the cytotoxic agent [206]. The tumor microenvironment is characterized not only by marked gradients in drug concentration but also by gradients in the rate of cell proliferation, all of which can influence tumor cell sensitivity to drug treatment [207]. Therefore we have concluded that the cells have developed a mechanism to better tolerate the presence of platinum in the cell. As noted in chapter 3, when CHD3 is transcriptionally silenced we see a decrease in cell proliferation. We predict that the role CHD3 plays in the regulation of cell growth allows the tumor cells to retain platinum drugs. Additionally, CHD3 may play a role in some other mechanism that allows it to escape and/or tolerate platinum therapy.

Our data provide novel insights to understanding the mechanisms of chemotherapy response, and may offer guidance to future experimentation and to developing novel approaches to overcome chemotherapy resistance in ovarian cancer cells. Further experimentation will be necessary to determine whether differentially expressed proteins play a role in influx and efflux of platinum drugs and whether they have a causal role in the chemotherapy response.

4.4 Methods

Measurement of Uptake and Efflux of c-14 Carboplatin in Ovarian Cells

HOSE 6-3 and HOSE 6-3 infected with the shRNA to *CHD3* were seeded at 10^5 cells in a volume of 500 µl of medium per well in a 24-well plate, 5 wells per cell type. After 24h the cells were washed with DMEM, no additives, and C^{14} carboplatin was applied at 1uCi in .3mL of DMEM, no additives, in 4 wells per cell type with the fifth well per cell type serving as a control and contained 300uL of DMEM with no additives. At time points 24h, 48h, 72h, and 96 hrs the cells were washed with PBS and harvested in a 100 µL suspension. For each time point a viability assessment of the cell suspensions was obtained with .4% trypan blue stain in PBS at a 1:1 concentration with 10uL of the suspension and counted with a hemocytometer. The cell suspensions were then spun at <10000g, washed 1x with PBS and repelleted. Cytoplasmic and nuclear fractions were prepared from the cells. The cell pellet was resuspended in 100 µL of buffer (150mM NaCl, 10mM Tris pH8.0, and 10% Nonidet P40 (NP40)). The suspensions were spun down, leaving a cytoplasmic fraction and the pelleted nuclei. The nuclei were washed with buffer, spun down in a microfuge and supernatant saved as the nuclear fraction. Uptake was determined by detecting the level of C^{14} in the extracts using a scintillation counter.

Efflux was assessed similarly to that of the influx with the exception that the start time point was 48 hrs *after* the initial treatment of the c-14 carboplatin. Time points of 0, 24, 48, 72, 96 hours were used to assess the efflux of carboplatin in the cells.

4.5 Figures

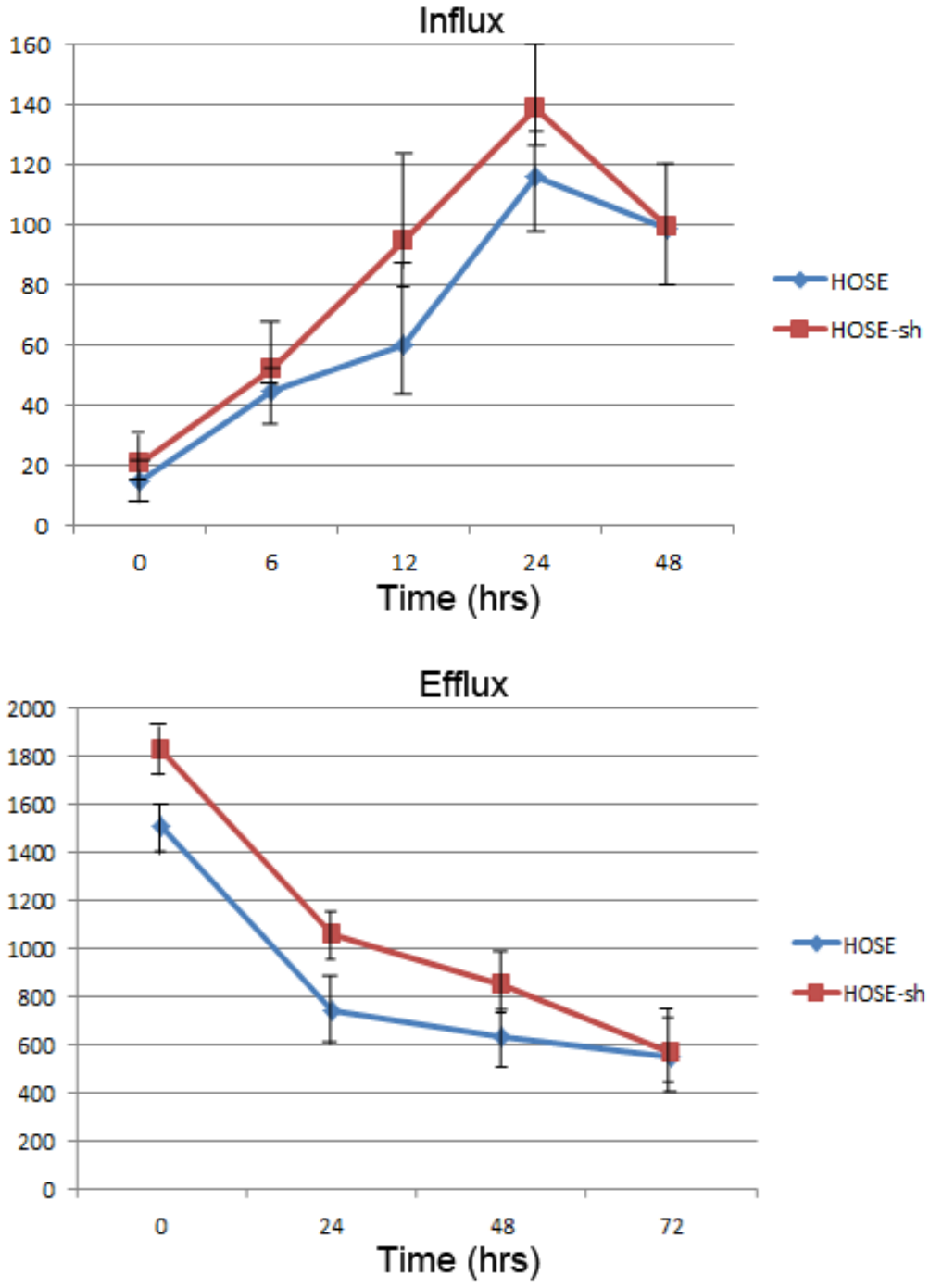


Figure 4-1 analyzes the effect of CHD3 suppression on the level of Carboplatin in the cell. The top panel is the influx of Carboplatin or the level of Platinum that enters the cell (HOSE

cell line in blue and HOSE shCHD3 cell line in red) as measured by the cpm of C_{14} labeled Carboplatin. The Y axis represents the cpm taken at different times (hrs on the X axis) normalized for the viable cell number. The bottom panel is the efflux of Carboplatin as measured by the cpm of C_{14} labeled Carboplatin. The Y axis and X axis are the same as above. As you can see, there is no significant change in platinum uptake or efflux in the Hose 6-3 cell lines with and without the shRNA to CHD3.

Table 4-1 Possible Reasons for Chemotherapy Resistance

Mechanism	Example
Alteration of target	Mutations, amplifications in drug targets rendering them inactive
Efflux/Influx	Overexpression of drug transporters, *MDR1* gene, P-glycoprotein efflux pump
DNA Repair/Tolerance	Upregulation of DNA repair mechanisms, XPB, XPA, ERCC1

[131, 208, 209]

Chapter V: Platinum induced apoptosis is decreased in *CHD3* silenced cells

5.1 Introduction

The primary cytotoxic effect of many chemotherapy drugs is mediated by apoptotic response in tumor cells. Apoptosis is a process of programmed cell death where there is an upregulation of pro-apoptotic factors or tumor suppressors and also a modulation of cell survival factors [210]. These genes may play a central role in a cells resistance to chemotherapy.

A number of genes involved in either the induction or inhibition of apoptosis, namely the p53, Akt, and phosphoinositol-3-OH-kinase (PI3K) gene families, are aberrantly regulated in ovarian cancer [211]. Because of their wide-ranging biological effects, deregulation of one or more of these factors may give rise to a failure of drug-induced apoptosis. p53 is a transcription factor associated with cell cycle regulation and the ability to induce apoptosis by turning on pro-apoptotic genes. The development of cancer can be blocked through the action of p53, which causes cells to enter quiescence or apoptosis [212]. EOC frequently exhibits mutations and alterations in p53 protein. Moreover, Cheng *et al*. (2002) suggest that the X-linked inhibitor of apoptosis proteins (Xiap), an intracellular anti-apoptotic protein, plays a key role in cell survival by modulating death signaling pathways and is a determinant of platinum resistance in ovarian cancer cells in vitro [210]. Changes in the expression of cell death and cell survival genes lead to activation of caspase-3. Specifically, in human ovarian cancer cells, chemotherapy agents such as cisplatin can up-regulate cell death genes (Fas and FasL), resulting in activation of caspase-8 and -3 and induction of apoptosis.

Analogously, in chemoresistant cells, cisplatin failed to up-regulate FasL or induce apoptosis [213].

Apoptotic cell death plays an important role in tissue development and homeostasis as well as in cancer cell clearance [214]. Current cancer therapies primarily exert their antitumor effect by triggering apoptosis in cancer cells. However, many malignant cells develop defects in the regulation of genes that control apoptosis, rendering them resistant to the induction of apoptosis by a wide variety of stimuli, including chemotherapeutic drugs [215-218]. Therefore, understanding the role that apoptosis pathways play in chemoresistance is crucial.

5.2 Results

5.2.1 *CHD3* resistance to platinum does not trigger apoptosis

Interestingly, cells with the *CHD3* shRNA do not fill in the plate as quickly as the parent cell line (with empty vector) as determined by MTT assay (**Figure 3-2 Panel A**) and by BrDU uptake assay (**Figure 3-2 Panel B**). This data demonstrates that the cells are cycling slower and therefore this could be a sign of increased cell death. To determine if there was a difference in well characterized p53 dependent apoptotic mechanisms in response to loss of *CHD3* we tested this by assaying two different markers of apoptosis, caspase 3 production and the accumulation of the γH2AX phosphorylation.

H2AX is a histone variant that is systematically found and ubiquitously distributed throughout the genome. Since it has been reported that DNA double-strand

breaks (DSBs) induce phosphorylation of H2AX at serine 139 (γH2AX), an immunocytochemical assay with antibodies recognizing γH2AX has become the gold standard for the detection of DSBs [219-221]. H2AX has been shown to be involved in the maintenance of genomic stability in response to DNA DSBs [221]. Phosphorylated H2AX is referred to as γH2AX. Induction of γH2AX is a p53 dependent mechanism. To understand if apoptosis was playing a role in chemoresistance to platinum response we examined the phosphorylation of γH2AX by western blot. Western blot analysis of phosphorylated H2AX production in ovarian cancer cell lines demonstrates that after a 72 hour treatment with platinum drugs, production of phosphorylated H2AX was induced. As seen in **Figure 5-1 Panel A**, we observed that γH2AX phosphorylation following treatment of platinum drugs in positive UV-treated control and parental HOSE cells lines but this induction was not seen upon suppression of *CHD3*. Additionally, we performed western blots for the induction of γH2AX phosphorylation after the 12 day treatment cycle with platinum drugs. **Figure 5-1 Panel B** indicates that even after treatment with platinum drugs for 3 cycles there was no induction of γH2AX phosphorylation. Platinum-based chemotherapy forms DNA adducts which results in DNA damage. These results indicate that the silencing of *CHD3* may play a central role in avoiding apoptosis triggered by DSB after platinum based chemotherapy. Additionally, these results also indicate that although cells with the sh*CHD3* have a slow proliferation rate (**Figure 3-2**), it is not because due to apoptosis. They are just going through cell division slower.

Apoptosis can be brought about by a family of proteases known as the caspases, the activity of which is responsible for the organized destruction of the cell. Caspases are thought of as the primary apoptotic executors [222], which act in a cascade ultimately

leading to the cleavage of substrates that produce the characteristic features of apoptosis. Analysis of apoptosis by western blot of Caspase-3 revealed parallel results. As seen in **Figure 5-1 Panel C** absence of activated caspase 3 in ovarian cancer cell lines demonstrate that even after treatment with platinum drugs, cleavage of pro-caspase 3 did not occur. In the present studies, we have observed that pro-caspase-3 cleavage following treatment of platinum drugs only in our parental HOSE cells lines and staurosporine-treated positive control.

5.2.2 *CHD3* knockdown does not cause DNA fragmentation

Apoptosis is associated with the fragmentation of chromosomal DNA into multiples of the 180 bp nucleosomal unit, known as DNA laddering [223]. Isolated ovarian cell line eukaryotic DNA separated by gel electrophoresis and visualized using ethidium bromide demonstrates that after treatment with platinum drugs, DNA laddering did not occur. As seen in **Figure 5-1 Panel D**, we observed DNA laddering following treatment of platinum drugs only in staurosporin-induced positive control and the parental HOSE cells lines treated with platinum for 1 cycle. However, after 3 cycles of chemotherapy treatment, the parental cell lines exhibited miner DNA fragmentation. Interestingly, this laddering was lost with suppression of *CHD3* in both the one cycle and 3 cycle platinum treatment cell populations. From these results we can see that the decreased number of cells when shRNA to the *CHD3* gene is introduced is not caused by an increase in apoptosis.

5.2.3 The effect of sh*CHD3* on apoptotic phenotype

A common phenotype for apoptosis is a rounded cell with large spherical edges and cytoplasmic blebbing with large separate granular component. As seen in **Figure 5-2**, we observed typical apoptotic associated morphology changes in our parental cell lines following the 3 cycle treatment with platinum drugs whereas cells with the shRNA to *CHD3* did not undergo as extensive of a morphological change. Overall, the morphology of the cytoplasm and nuclear region was consistent with numerous and previous reports in the literature [224, 225]. Light microscope imaging was used to objectively characterize the morphology of these cells. Therefore, it appears that some of the cells may be undergoing apoptosis, but not as many as the control cell lines.

5.2.4 *CHD3* resistance to platinum does not cause cellular senescence

A possible explanation for the observed decrease in total number of cells in culture with the suppression of *CHD3* is induction of senescence. Cellular senescence is a phenomenon in cells whereby normal diploid cells lose their ability to divide normally and usually takes place after about 50 cell divisions [226, 227]. To determine cellular senescence in our parental and sh*CHD3* cell populations we used the Chemicon Cellular Senescence Assay kit to detect SA-β-GAL activity. In this assay, SA-β-GAL catalyzes the hydrolysis of X-gal, which results in the accumulation of a distinctive blue color in senescent cells. The cells can be easily viewed under a light microscope for

identification of SA-β-GAL activity. We obtained similar results to what we found for the analysis of apoptosis in although the Hose 6-3 sh*CHD3* cell population had a slight increase in senescence, none of our tumor cell lines did, relative to their parental controls. These results can be seen in **Figure 5-2**. These data show us that in tumor cells the loss of *CHD3* does not induce senescence considerable. This assay was repeated using the pLKO vector for *CHD3* and we obtained similar results (**Appendix 4**).

5.3 Discussion

A large set of genetic, functional and biochemical studies suggest that cancer cells become prosperous against a variety of chemotherapeutic drugs by exploiting their intrinsic resistance to apoptosis and by reprogramming their proliferation and survival pathways during progression. Chemotherapy remains the primary treatment for ovarian cancer. However, some tumors are inherently insensitive to chemotherapeutic agents and others acquire resistance upon relapse. Most conventional agents damage cellular components, often DNA, and for years it was assumed that this damage was directly responsible for their anti-tumor effect [228]. Apoptosis is a well-characterized post-damage program that contributes to drug action [228]. Diverse anticancer agents can induce apoptosis through common pathways and, consequently, mutations that disable these pathways can confer multidrug resistance [229]. For example, the Bcl-2 oncoprotein is a potent suppressor of apoptosis that can produce multidrug resistance in cultured cells and animal models. Furthermore, Bcl-2 overexpression correlates with

poor treatment outcome in some clinical settings [228]. Nevertheless, despite extensive efforts, the overall contribution of apoptotic defects to clinical drug resistance has been difficult to assess.

Platinum based drugs form DNA adducts, resulting in inter and intrastrand crosslinks. This ultimately leads to DNA damage, mainly by double strand breaks. Therefore, we hypothesize that one of the targets regulated by CHD3 and/or the NuRD complex is a DNA repair protein. Further experiments are needed to confirm this hypothesis. In our experiments, even after the 3 cycle treatment with chemotherapeutic drugs, cells with the shRNA to *CHD3* did not undergo apoptosis. Thus, *CHD3* deficient cells might be contributing to chemosensitivity by avoiding apoptosis. Furthermore, as indicated by the MTT and BrdU cellular proliferation assay (**Figure 3-2**), this avoidance of apoptosis is not due to growth arrest. This suggests that CHD3 may play a regulatory role in inhibiting apoptosis directly or indirectly through its interaction with genes in the apoptosis pathway. *TP53* accumulation plays a key role in induction of apoptosis. Ovarian carcinomas are characterized by *TP53* mutations in almost all tumors (96%), which may suggest one reason for the failure to activate apoptosis in the chemoresistant population of cells with CHD3 suppressed [133].

In addition to apoptosis, cancer cells have also been shown to be effectively eliminated after DNA damage by necrosis, mitotic catastrophe and autophagy [230, 231]. Necrosis may be triggered by the rupture of the plasmatic membrane and may be accompanied by formation of an inflammatory process [232]. On the contrary, apoptosis involves a cleaner type of death, in which the chromatin is condensed; the DNA becomes fragmented forming vesicles known as apoptotic bodies. These are rapidly phagocytosed

by the macrophages with the result that the cell disappears without any inflammatory phenomena [233]. Cytotoxic agents have been shown to play a critical role in killing of tumor cells in response to cytotoxic agents by inducing apoptosis however, the significance of apoptosis as the main mechanism of cancer cell death in response to anticancer chemotherapy has also been challenged [230]. Thus, nonapoptotic modes of cell death, such as necrosis, may also be crucial in response to cytotoxic therapy. It will be interesting to examine the role of necrosis in chemoresistant sh*CHD3* cells in the lab.

Furthermore, apoptosis can be triggered by p53 independent pathways as well. One such pathway is the p73-mediated apoptosis in response to DNA damage. The expression of the *TP73* gene is frequently altered in cancer and its modulation enhances cancer cell sensitivity to drug-induced apoptosis [234, 235]. Recent research has established that *TP73* mRNA accumulates in human cell tumor lines in response to DNA damage induced by various agents, including the chemotherapeutic drugs etoposide, camptothecin, daunorubicin and doxorubicin [236]. DNA-damage checkpoint kinases, *CHK1* and *CHK2* are essential for the induction of cell-cycle arrest, DNA repair and apoptosis. Specifically, in ovarian cancer, deregulation of this pathway may confer resistance to platinum based drugs. The role that this pathway may play in ovarian cancer chemoresistance is the basis for future research in the lab.

5.4 Methods

Immunoblotting.

Samples were harvested from the different cell lines, total protein was extracted and protein concentration was determined using the Bradford Assay (Bio-Rad) as per manufacturer's instructions. Western blotting was performed using 25 μg of protein, according to standard procedures using the Invitrogen NuPAGE® Novex system. Immunodetection was done using anti-mouse monoclonal antibody against Phosphorylated H2AX and Caspase-3 (Cell Signaling), diluted 1:1,000, followed by incubation with anti-rabbit secondary antibody (horseradish peroxidase HRP-conjugated) (Cell Signaling), diluted 1:10,000, for chemiluminescent detection. Anti-mouse monoclonal Tubulin (HRP-conjugated) (Cell Signaling), diluted 1:50,000 was used as control. The Western Lightning ECL-plus detection system (Perkin Elmer) was used for signal detection. Staurosporine (STS, Sigma) was dissolved in DMSO was made up in a stock solution of 1 mM. Positive control NIH 3T3 cells were STS treated overnight at 37°C and harvested for protein extract. UV treated NIH 3T3 cell lysate was also used as a positive control (Cell Signaling).

Senescence-associated β -galactosidase (SA-β-Gal).

Ovarian cancer cell lines were examined for senescence using the Chemicon's Cellular Senescence Assay Kit. SA-β-gal (or senescence associated β-gal) is a hypothetical hydrolase enzyme that catalyzes the hydrolysis of B-galactosides into monosaccharides only in senescent cells. The blue-dyed precipitate results from the cleavage of the chromogenic substrate X-Gal. Cells were fixed and stained according to

the manufacturer's protocol. Briefly, cells growing in culture were fixed at room temperature for 2 h in 4% paraformaldehyde/PBS and then washed 3 times in 1× PBS. Cells were incubated for 4 h at 37°C with β-Gal staining solution (KAA002; Chemicon) as recommended by the vendor. All cells were photographed under the same conditions, and β-Gal activity in each animal was quantified by examining at least 10 fields of view.

Detection of Nucleosomal DNA Fragmentation

DNA was isolated from all cell lines using QIAgen DNEasy kit after 3 and 12 days treatment with carboplatin. Positive controls consist of UV treated NIH 3T3 cells. Samples were subjected to electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.

Detection of Morphological Features Associated with Apoptosis

Morphology characteristics were examined in the ovarian cell lines with and without the shRNA to *CHD3* after carboplatin treatment at the IC50 value for 72 hours. Cells were examined under a light microscope for cellular appearance and morphology consistent with cell death and proliferation. Specifically, non-apoptotic cells contained intact nuclei with little to no condensed features as well as no blebbing, extensive vacuolization, or condensation of cytoplasmic or nuclear contents.

5.5 Figures
Figure 5-1

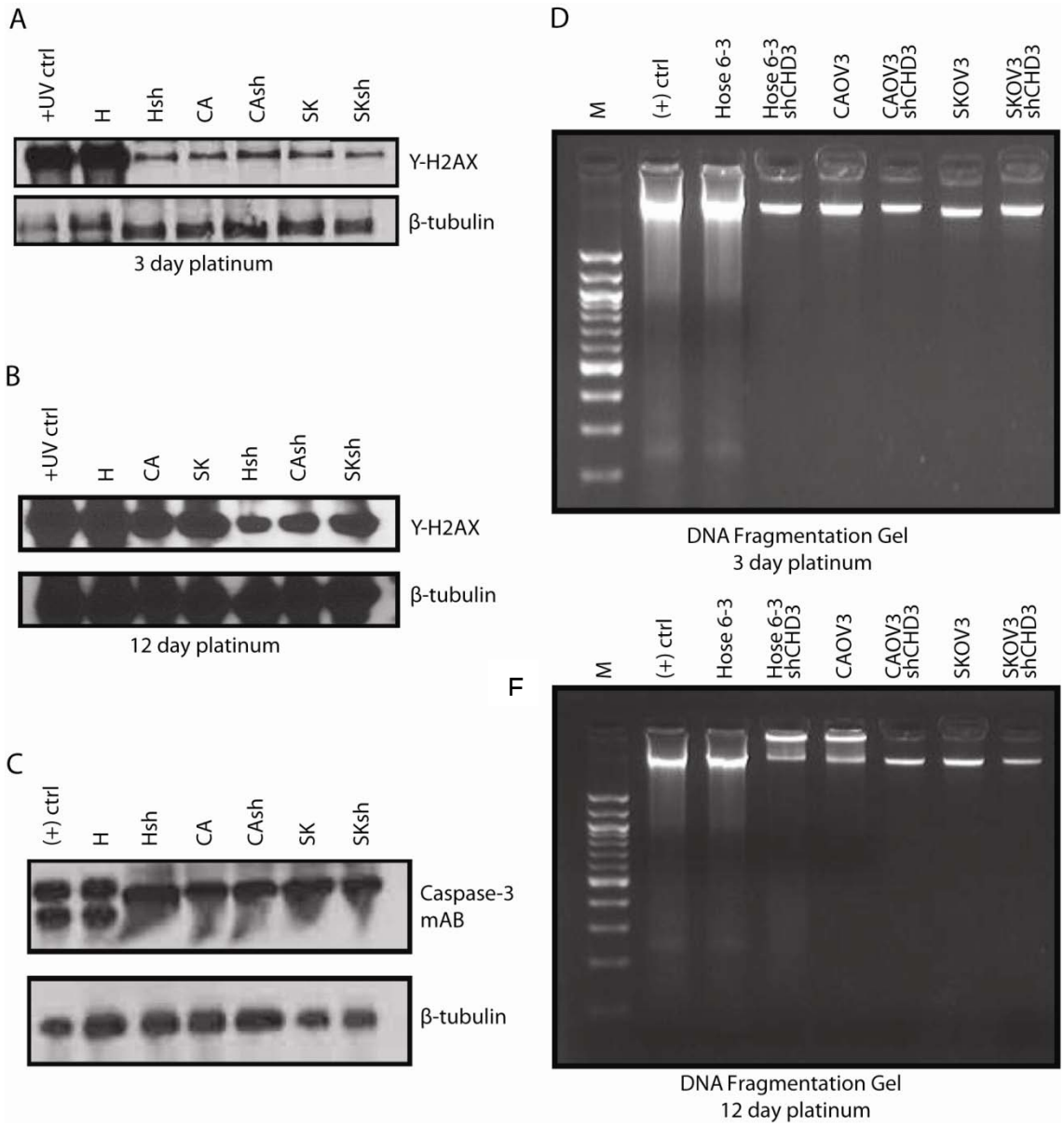


Figure 5-1: *CHD3* Associated Resistance to Platinum Does Not Cause Apoptosis in our Ovarian Cell Lines**. Panel A-C** are western blots. **(A-B)** The top panel shows the western blot of phosphorylated H2AX production in ovarian cancer cell lines. The blot was done after a 3 day **(A)** and 12 day **(B)** treatment with platinum drugs. UV-treated NIH3T3 cell line was used as a positive control. As you can see from the blot, phosphorylation of H2AX occurs after treatment of the control UV-treated cells and Hose 6-3 cells after 3 days platinum treatment. After 12 days platinum treatment, phosphorylated H2AX is more profound in the parental cell lines but not the cells with the CHD3 silenced. **Panel C** represents pro-caspase-3 cleavage following 12 day treatment of platinum drugs. Staurosporine treated NIH 3T3 was used as the positive

control. The bottom panels are the β-tubulin loading control. Above the blot are the names of the cell lines used in this experiment. As you can see from the blots, caspase-3 is cleaved after treatment with platinum in the positive control treated cells and the Hose 6-3 parental cell line only. **Panel D-E** represents isolated ovarian cell line eukaryotic DNA separated by gel electrophoresis and visualized using ethidium bromide. The gels represent DNA fragmentation after 3 day **(D)** and 12 day **(E)** treatment with platinum drugs. UV-treated NIH3T3 cell line was used as a positive control. The cell lines used are listed above the blot. The gel represents DNA fragmentation in the positive control treated cell line as well as the Hose 6-3 parental cell line. Cell lines silenced for CHD3 do not show a typical DNA smear even after 12 days of platinum treatment.

Figure 5-2

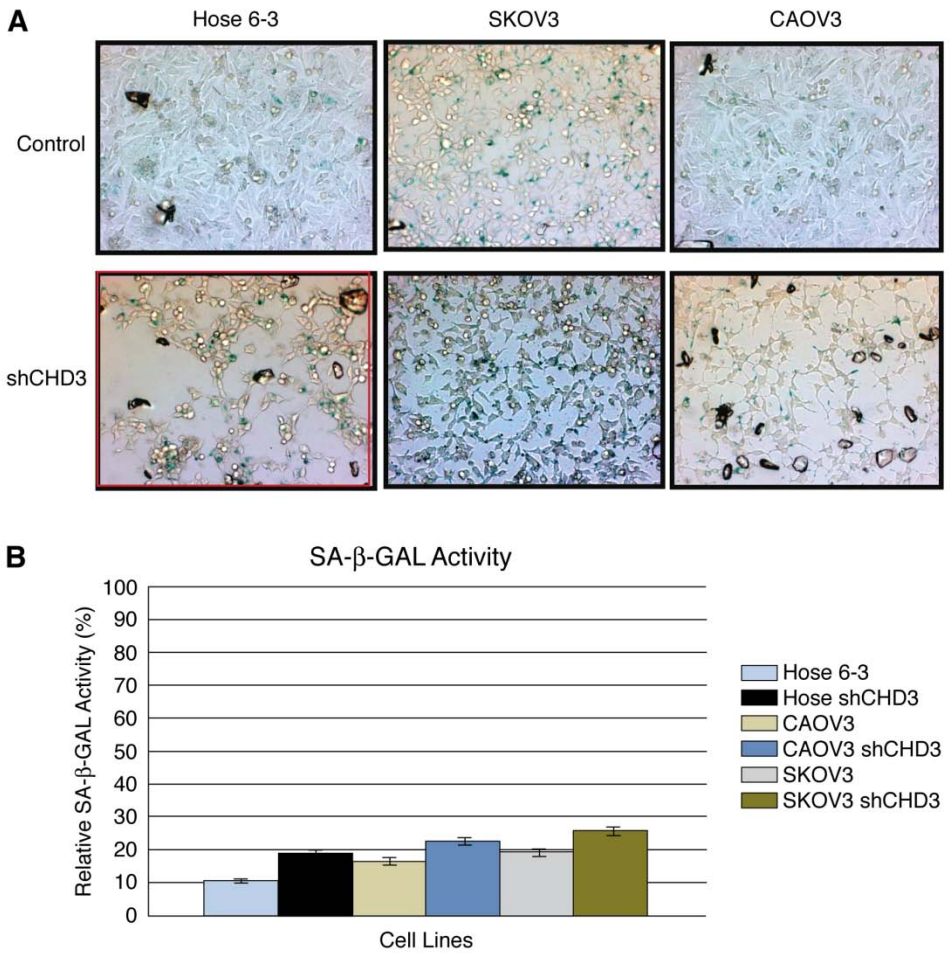


Figure 5-2: Platinum-resistant *CHD3* cells do not have a significant change in senescence. **Panel A** image represents SA-β-gal staining of the EOC cells with and with sh*CHD3* (Scale bar, 100 μm.). Top panels represent the control cell lines and the bottom panels represent shRNA infections with *CHD3*. Field of view under a phase contrast microscope using 40x magification. The data shown represents a representative view. **Panel B** represents a bar graph quantifying cellular senescence in the parental cell lines compared to the shCHD3 silenced cell lines. Error bars represent standard deviation

(n=5; $P<0.05$). Cell lines are listed on the x-axis and the y-axis represents the relative SA-β-gal cell counts over at least 10 fields of view. As you can see from the graph, there is not a significant change in cellular senescence as determined by this assay.

Figure 5-3

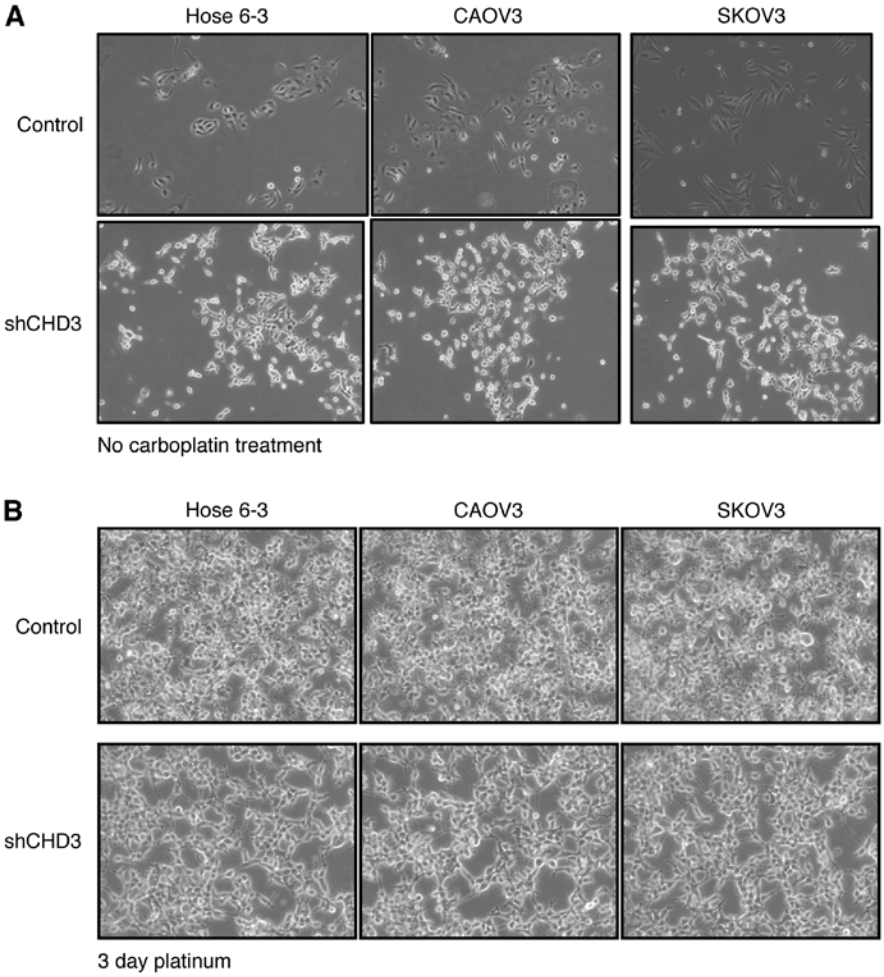


Figure 5-3 are light microscope pictures depicting apoptotic phenotype after before (A) and after treatment (B) with platinum drugs. The cells treated for 72 hours at the IC50 dose. As you can see from the images, there appears to be cellular changes in both the parental and shCHD3 cells after platinum treatment, which may be associated with an increase in apoptosis of the cells.

Chapter VI: Suppression of *CHD3* induces mesenchymal-like features and an increased metastatic potential.

6.1 Introduction

Development of distant metastases is a later stage of cancer progression and is responsible for the majority of cancer related deaths [2]. While clinically of great importance, the biology of metastasis remains unresolved. The process of tumor metastasis consists of multiple steps which include first, the escape of cancer cells from the tumor site and then invasion into the stroma and entrance into blood circulation or lymphatic system. Finally, distal colonization requires that cancer cells invade and grow in the new environment at the distant sites they have colonized [237].

Recently, the process of epithelial-mesenchymal transition (EMT), originally identified in the field of embryology, has been extended to cancer progression and metastasis [238, 239]. Both in vitro and in vivo models support the role of EMT in metastasis. EMT associated markers can be screened in tumor samples. These include mesenchymal specific markers such as Vimentin, fibronectin, and N-cadherin [240], epithelial specific markers such as E-cadherin and cytokeratin [241], and transcription factors such as SNAIL, SLUG and TWIST [242].

The process of EMT consists of multiple steps. First, cell to cell adhesion disintegrates with the loss of epithelial markers such as E-cadherin and the gain of mesenchymal markers such as Vimentin and/or N-cadherin. Next, there is a loss of

basoapical polarization and the acquisition of front-rear polarization. Then, the cytoskeleton undergoes remodeling, with changes in cortical actin and actin stress fibers. Finally, cell-matrix adhesion is altered, with activation of proteolytic enzymes such as matrix metalloproteases [243]. Similarly, that the process of metastasis in epithelial ovarian cancer also consists of multiple steps which include detachment from the primary tumor and invasion into the surrounding stroma [68]. This enables the cancer to enter blood circulation and invade distant sites. Thus, the process of EMT during cancer progression closely resembles that of embryological development [244].

While there is an association of EMT with resistance, the mechanisms are presently unclear. Cells undergoing EMT become invasive and develop resistance to anticancer agents. For instance, upregulation of TWIST was associated with cellular resistance to paclitaxel in human nasopharyngeal, bladder, ovarian, and prostate cancers [245]. Oxaliplatin resistant cell populations of colorectal cancer can acquire the ability to migrate and invade with phenotypic changes resembling EMT. These changes consist of spindle shape appearance, loss of polarity, intercellular separation, and pseudopodia formation [246]. Furthermore, in pancreatic and ovarian cancer, stable cell lines resistant to gemcitabine and paclitaxel established by continuous exposure can undergo EMT with increased expression of the EMT transcription factor markers Snail and Twist [247, 248]. Additionally, EMT appears to be responsible for conferring resistance in carcinomas treated with targeted therapy. For example, lung cancer cell lines that have undergone EMT (expressing Vimentin and/or fibronectin), were insensitive to the growth inhibitory effects of EGFR kinase inhibitors (erlotinib) in vitro and in xenografts [249] as well as other EGFR inhibitors such as gefitinib and cetuximab [250, 251]. Clinically, patients

who are initially responsive to anticancer treatments often relapse, even after they were initially sensitive to treatments. Therefore, we hypothesize that chemoresistance and the subsequent rapid progression of a tumor appears to be associated with EMT.

Clinically, EOC frequently shows sudden and multiple metastasis following continuous treatment with chemotherapeutic agents, including platinum based drugs. Our experiments indicate that when ovarian cancer cell lines are silenced for *CHD3* expression, an EMT-like phenotype is seen. Furthermore, these cells display enhanced motile and invasive behavior in vitro compared to their parental counterparts. Our data demonstrates that development of platinum resistance in EOC cells is accompanied by a transition from an epithelial to a mesenchymal phenotype.

6.2 Results

6.2.1 *CHD3* downregulation confers a tumorgenic phenotype

Since we have established that cells transcriptionally suppressed for *CHD3* tolerate platinum we wanted to further characterize if the loss of *CHD3* causes any other changes to the cells. We first addressed if *CHD3* had any features of a tumor suppressor, by assaying if loss of *CHD3* had transformative properties. We first assayed whether cells harboring the shRNA to *CHD3* lost anchorage independence, and performed experiments for growth in soft agar, an *in vitro* surrogate measure of tumorigenicity [252, 253]. Generally this assay is performed with immortalized cells. When shRNA for *CHD3* was introduced into HOSE cells and plated in soft agar, many colonies grew with almost no

colonies formed from the cells with the empty vector control (**Figure 6-1**). When the tumor cell lines with empty vector were plated with agar as expected, because they are tumor cell lines, colonies formed. However, when the shRNA for *CHD3* was introduced into the tumor cell lines there was a marked increase in the number of colonies that formed. The colonies that formed were somewhat smaller than those formed in the control but there were many more (**Figure 6-1**).

6.2.2 *CHD3* downregulation is associated with increased motility and invasiveness

Cell migration is a highly integrated multistep process that orchestrates embryonic morphogenesis, contributes to tissue repair and regeneration, and drives disease progression in cancer [254]. Epithelial cells with enhanced migratory and invasive potential have been implicated in many physiological and pathological processes. The promotion of both cell migration and invasion is thought to play a major role in EMT [255]. The *in vitro* scratch assay is a straightforward and economical method to study cell migration *in vitro*. This method is based on the observation that, upon creation of a new artificial gap, so called "scratch", on a confluent cell monolayer, the cells on the edge of the newly created gap will move toward the opening to close the "scratch" until new cell–cell contacts are established again. **Figure 6-1** shows images that were captured at time interval 0 and 16 hours to determine the rate of migration. In-vitro patterns of migration of epithelial cells mimics the behavior of these cells in vivo and allows study of cell-cell interactions as well as cell interaction with the extracellular matrix [256]. Our results demonstrate that the suppression of *CHD3* increased migratory potential of the parental

control ovarian cells. Similarly, these results were also confirmed in the pLKO *CHD3* vector and can be seen in **Appendix 5-2, Panel A-B**.

We next determined if loss of *CHD3* had an effect on the invasive potential of the cells, or does suppression of *CHD3* increase migration. To determine this we utilized Matrigel invasion chambers and compared cells with *CHD3* shRNA to cells with empty vector. Invasion of tumor cells into Matrigel has been used to characterize involvement of extra-cellular matrix receptors and matrix degrading enzymes which play roles in tumor progression [257]. From **Figure 6-2 Panel C** it is clear that suppression of *CHD3* markedly increases the migration of cells into matrigel. In fact even in the tumor cell lines the migration increases. The quantification for the assay is shown in **Figure 6-2 Panel D**. Our results suggest that a decrease in the level of *CHD3* is associated with increased invasiveness compared to the non-silencing controls. Additionally, this assay was repeated in the pLKO vector for *CHD3* and we obtained similar results (**Appendix 5-1**)

.

6.2.3 *CHD3* silencing confers an epithelial to mesenchymal transition

We noticed that the cells with the shRNA for *CHD3* changed phenotypically as compared to the empty vector parent cell lines (**Figure 6-3**). The typical epithelial uniform monolayer of the HOSE cells disappeared and although the tumor cell lines do not make a typical epithelial layer the uniformity normally present disappeared upon suppression of *CHD3*. Instead, the cells had an irregular shape that was not uniform in composition or density. The cells had a more extended and elongated shape, relative to

the empty vector parent cell lines, a phenotype typical of transition of epithelia to mesenchyma (EMT) [258]. Therefore, we tested several molecular markers for EMT: N-cadherin, E-cadherin, Vimentin, and transcription factors such as Snail1 (Snail), and Twist (see **Table 6-1**). If the cells have undergone EMT we expect to see an increase in the level of N-cadherin, Vimentin and Twist, and expect a reduction in the levels of E-cadherin. We first tested the expression of these genes by RT-Q-PCR (**Figure 6-4 Panel A)** demonstrating that the message levels are consistent with the cells undergoing EMT. We then analyzed the protein level of these markers by western blotting to determine if the expression correlated with the protein level and found they were in complete concordance (**Figure 6-4 Panel B**). RT-Q-PCR experiments for the EMT molecular markers were repeated using the pLKO vector and the same results were obtained (see **Appendix 5-3)**. Therefore, we conclude that the cells are undergoing an EMT.

Thus it appears that phenotypically and by molecular markers the suppression of *CHD3* causes the epithelial cells to transition to a mesenchymal like state, and that this may explain the increased resistance that has been shown to be involved in other cancers [243, 259-261]. In conclusion, we determined that the silencing of the chromodomain-helicase-DNA-binding protein 3 was sufficient to activate a complex program that lead to acquisition of mesenchymal-like morphology, increased motility and invasion ability, and increased platinum resistance. Overall, these results suggest that the shRNA to *CHD3* induced metastatic potential in-vitro

6.3 Discussion

Transformation at cellular level refers to the collective changes, such as uncontrolled cell division and morphological alterations, which occur to convert a normal cell to cancer cell [262]. Anchorage independent growth of cells is defined further by the ability of cells to proliferate in a semisolid medium [263]. Clinically, chemoresistance and enhanced metastatic ability are frequently concurrent during therapeutic course of EOC and may be linked. Therefore we tested the ability of *CHD3* suppression to have transformative properties in the ovarian cell. The ability of cells to form colonies in soft agar is considered to be a transition state of a normal cell to a transformed cell. In the immortalized epithelia suppression of *CHD3* clearly increased colony formation in the agar. Interestingly when the tumor cell lines which form colonies in soft agar normally where used for the assay, there was an increase in colony formation. However the colonies were smaller, with many very small colonies. Furthermore, this phenotype seems to be in contrast to the two-dimensional cell culture phenotype where we saw a decrease in cell proliferation from cells harboring the shRNA to CHD3. We hypothesize this is because CHD3 has a role in three dimensional architecture. This can be somewhat explained by a possible role that CHD3 might play in altering cell adhesion molecules allowing cells to form colonies in soft agar. The cell culture in vitro system does not entirely reflect the natural development of tumors. More conclusively, when cells with shCHD3 are injected subcutaneously into nu/nu mice, we see a rapid formation of tumors in these mice (see **Appendix 5-4**). Therefore, we predict CHD3 plays a role in three-dimensional morphology.

A possible interpretation of the numerous small colonies was that the suppression of *CHD3* is in part required for the loss of contact growth and is involved in migration, another feature of tumorigenesis. We therefore tested the invasive potential of the cells with *CHD3* loss. We found that all cells with *CHD3* suppression had increased invasive potential by this assay. We also replicated these results with the scratch assay. One of the major advantages of the simple wound healing method is that it mimics to some extent migration of cells *in vivo* and is suitable for measuring migration of population of cells [264]. We have analyzed the expression of the *CHD3* gene in these tumors as well as in publicly available data and while CHD3s expression on average is lower in tumor than normal tissue we did not observe a correlation of *CHD3* suppression with metastasis as detailed in the clinical data. However, it is our hope CHD3 with more accurate informatics analysis we will identify CHD3 as a potential biomarker for EOC.

Overcoming intrinsic and acquired chemoresistance is the major challenge in treating ovarian cancer patients. Molecular and phenotypic associations between chemoresistance and the acquisition of epithelial-mesenchymal transition (EMT)–like phenotype of cancer cells has recently emerged (reviewed in [68, 260, 265, 266]. For example, in colorectal cancer oxaliplatin-resistant cells underwent EMT [246]. Interestingly, in paclitaxel-resistant ovarian cancer cells, Kajiyama report phenotypic changes consistent with EMT with decreased expression of the epithelial adhesion molecule E-cadherin and an increase in mesenchymal markers such as Vimentin, α-SMA, and fibronectin [267]. Recently, phenotypic changes associated with EMT such as a spindle-cell shape and increased pseudopodia formation was reported in gefitinib-resistant lung cancer cells [268, 269]. Additionally, Rho et al. saw a decrease in the

expression of E-cadherin and an increase in the expression of the mesenchymal marker Vimentin [38]. In pancreatic cancer cells, recent reports suggest that gemcitabine-resistant cells acquired EMT characteristics through the Notch signaling pathway [83]. Collectively, these studies clearly provide strong evidence linking chemoresistance to EMT, and provide a plausible argument for EMT as a mechanism of chemoresistance in EOC. We predict *CHD3* to be a major player in this mechanism.

Cancer cells undergoing EMT can acquire invasive properties and enter the surrounding stroma, resulting in the creation of a favorable microenvironment for cancer progression and metastasis. Thus, EMT could be closely involved in carcinogenesis, invasion, metastasis, recurrence, and chemoresistance [270]. Previous results have indicated that EMT-associated genes *Twist, Fibronectin,* and *Claudin-1* play a role in EMT in ovarian cancer [270]. Upregulation of *Twist* is associated with cellular resistance to paclitaxel in human ovarian cancer [271]. More specifically, results indicate that ovarian cancer cell lines undergoing continual treatment with anticancer drugs can undergo EMT with increased expression of *Snail* and *Twist*, EMT-regulatory transcription factors. Our results indicate that when *CHD3* is repressed, the morphology of these cells change. In contrast to the parental cell lines, cells with *CHD3* repressed clearly begin to lose their epithelial features and acquire morphologic and molecular alterations consistent with EMT and a more mesenchymal phenotype. That is, there is dissolution of cell–cell contacts and loss of apico–basolateral polarity, resulting in the formation of migratory mesenchymal cells with invasive properties. Again, this provides further evidence for a direct role for *CHD3* in chemoresistance through EMT, perhaps as

a regulator of the EMT molecular markers. It will be important to study the interaction of *CHD3* with these markers in the future.

Increased invasion, migration through a basement membrane, and transformation in soft agar, as well as the phenotypic changes we noted in vitro all suggested that cells with *CHD3* repressed are undergoing epithelial to mesenchymal transition. More conclusively, using RT-Q-PCR and Western Blot we looked at the commonly used molecular markers. Our results indicate that these striking morphological features were associated at the molecular level with an increased expression of the mesenchymal protein and transcripts Vimentin, N-cadherin and Twist-1 and a decreased expression of the epithelial marker E-cadherin (for EMT markers, see **Table 6-1**). We predict that the epigenetic silencing of *CHD3* plays a main role in the transition of epithelial cells to mesenchymal cells. This silencing of this chromodomain helicase protein may result in the transcriptional silencing of other key players in EMT and this transition enables the cell to escape chemotherapy treatment. It will be interesting to follow up with this prediction in the future.

In conclusion, we determined that the silencing of the chromodomain-helicase-DNA-binding protein 3 was sufficient to activate a complex program that lead to acquisition of mesenchymal-like morphology, increased motility and invasion ability, and increased platinum resistance. Overall, these results suggest that the shRNA to *CHD3* induced metastatic potential in-vitro. Our current results establish a link between chemoresistance and the epigenetic induction of EMT.

6.4 Methods

Invasion and Migration Assay

We carried out a transwell invasion assay to evaluate the invasive potential of the cell lines (Hose 6-3, CAOV3, SKOV-3), which were successfully transfected with *CHD3* shRNA or controls (mock and non-silencing control). Transwell invasion assay experiments were carried out according to the manufacturer's instructions (BD Bioscience). Briefly, transwell inserts with 8-μm pores coated with Matrigel and used as an invasion chamber. Cell suspension (500 μl; 1×10^5/ml) was added to the upper chambers. The same medium of 750 μl were placed in the lower wells and incubated for 24 h. Cells that had invaded to lower surface of the Matrigel-coated membrane were fixed with 4% methanol in PBS, stained with H&E, and counted in five random fields under a light microscope. Cells that invaded the lower surface of the Matrigel-coated membrane were fixed and stained using toluene blue in 2% sodium carbonate. The number of invading cells was quantified and normalized to mock-transfected controls. Data are representative of 3 independent experiments (biological replicates). For migration, a scratch/wound healing assay was used. Briefly, using a p20 tip a "scratch" was made on a confluent monolayer of cells, creating a gap. If cells are able to migrate, they can move toward the opening to close the gap forming new cell-cell contacts. Images were captured at time interval 0 and 16 hours to determine the rate of migration. Quantification of the area enclosed by the scratch after 16 hours was done using ImageJ software.

Soft-Agar Foci Formation Assay

Hose 6-3 and CAOV3 ovarian cell lines (2.5 cells/dish) containing *CHD3* shRNAs or non-silencing controls were plated to 60mm dishes on a 1.0 ml layer of 0.7% (w/v) bacto-agar (BD Bioscience) reconstituted in serum-containing medium. Cells were then covered with 0.75 ml of 0.35% (w/v) warm agar in serum-containing medium. Cells were cultured for two weeks and cellular foci were photographed and foci number was quantified.

Immunoblotting

Samples were harvested from the different cell lines, total protein was extracted and protein concentration was determined using the Bradford Assay (Bio-Rad) as per manufacturer's instructions. Western blotting was performed using 25 μg of protein, according to standard procedures using the Invitrogen NuPAGE® Novex system or the Invitrogen E-Base Integrated Device. After electrophoresis, the proteins were transferred electrophoretically to a PVDF membrane (Millipore, Bedford, MA). After blocking (Sigma), the membrane was incubated overnight at 4C in primary antibody at the recommended dilution [(*CHD3*, E-cadherin, N-cadherin, Vimentin (Cell Signaling) Twist (Santa Cruz Biotechnology)]. The membrane was washed four times with Tween/PBS for 15 minutes each time, and then incubated with the appropriate secondary antibody

diluted 1:10,000, [horseradish peroxidase HRP-conjugated (Cell Signaling)] for 1-2 hours. After washing again four times in Tween/PBS, the membrane was treated with the Western Lightning ECL-plus detection system (Perkin Elmer) for signal detection. Anti-mouse monoclonal β-Tubulin (HRP-conjugated) (Cell Signaling), diluted 1:50,000 was used as control. Quantification of protein lysates was done using ImageJ, a public domain Java based image processing program, available for download at http://rsbweb.nih.gov/ij/

6.5 Figures

Figure 6-1

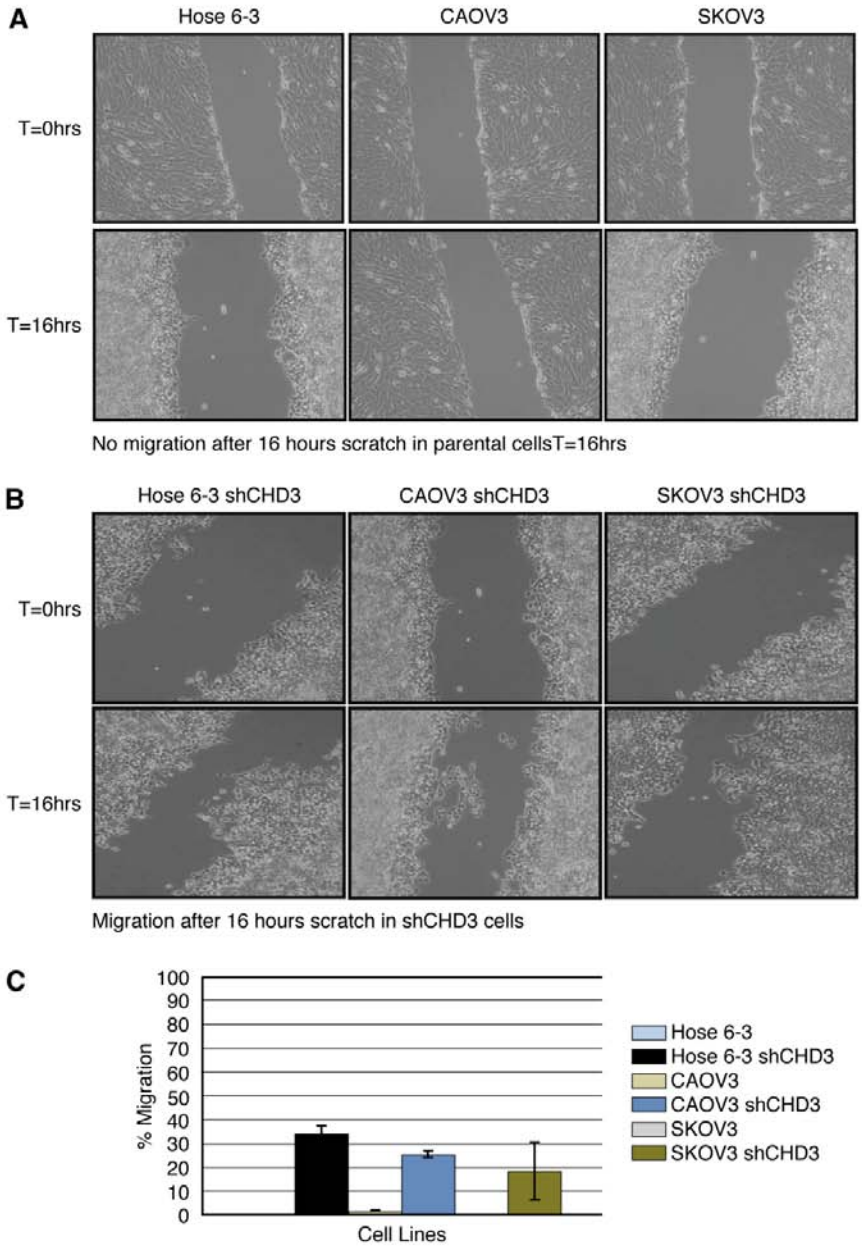


Figure 6-1 shCHD3 silencing confers a migratory phenotype. **Panel A** shows the results of a scratch assay for demonstrating migratory potential. A scratch is made in the cell monolayer (time = 0 h) over 16 hours. Cell images were taken under phase-contrast microscopy. Images in **Panel A** represents images from the control cell lines and **Panel B** is *CHD3* suppressed cells are represented on the bottom two panels. **Panel C** represents the migration quantification from the scratch assay. Quantification was determined using ImageJ. The area was measured for each scratch and the parental was compared to the shCHD3 cell lines. Percentages are an average of 3 independent experiments and error bars represent the standard deviation ($n=3$, $p<0.05$).

Figure 6-2

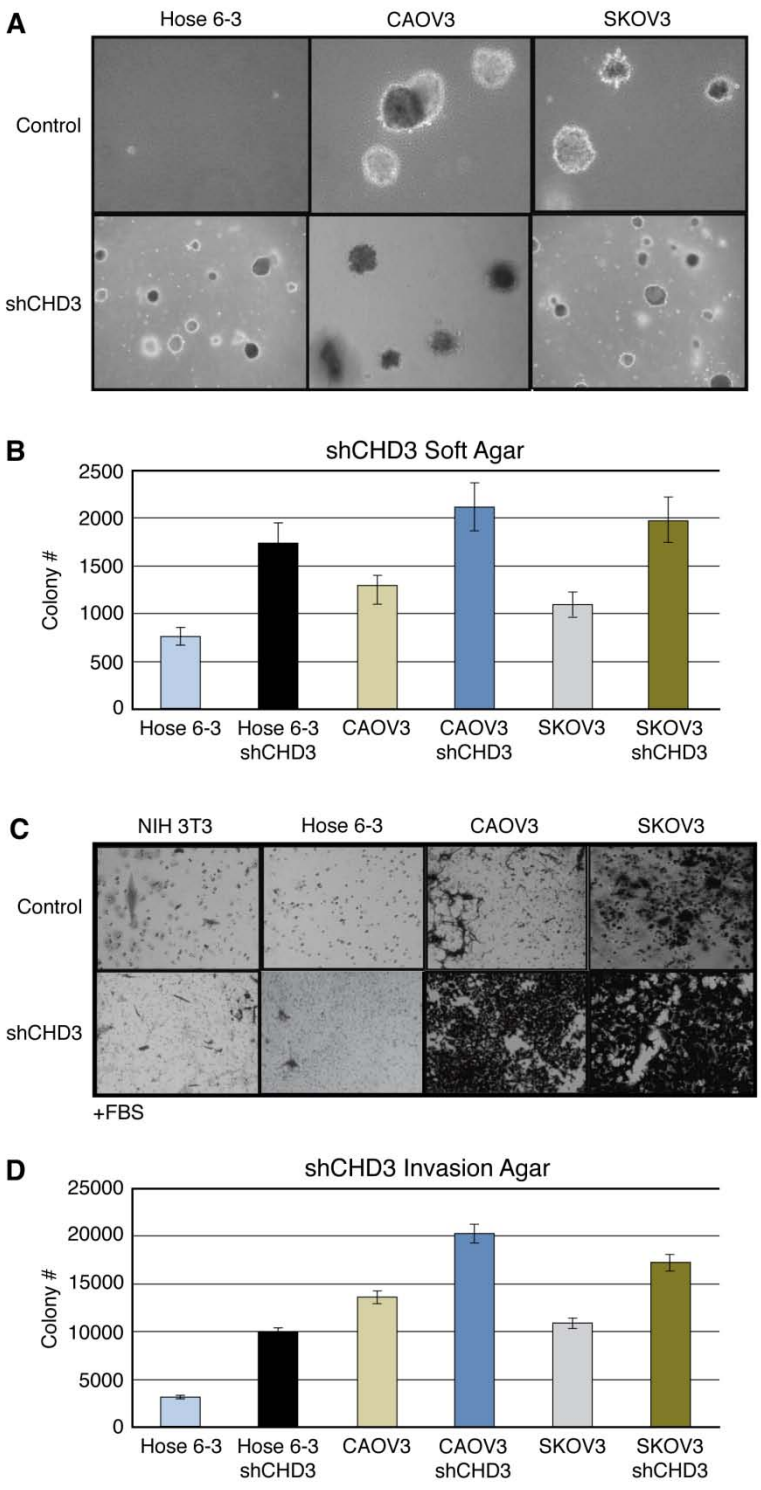

A
Hose 6-3
CAOV3
SKOV3
Control
shCHD3
B
shCHD3 Soft Agar
Colony #
2500
2000
1500
1000
500
0
Hose 6-3
Hose 6-3 shCHD3
CAOV3
CAOV3 shCHD3
SKOV3
SKOV3 shCHD3
C
NIH 3T3
Hose 6-3
CAOV3
SKOV3
Control
shCHD3
+FBS
D
shCHD3 Invasion Agar
Colony #
25000
20000
15000
10000
5000
0
Hose 6-3
Hose 6-3 shCHD3
CAOV3
CAOV3 shCHD3
SKOV3
SKOV3 shCHD3

Figure 6-2: demonstrates that suppression of *CHD3* induces tumorigenicity and increases invasiveness. **Panel A** is phase contrast microscopy of the soft agar colony formation assay with control cell lines (top images) and *CHD3* suppressed cell line (Bottom images) **Panel B** is a bar graph that represents the colony formation assays done in triplicate; $p < 0.05$ (Student t-test). The x-axis represents the cell lines and the y-axis represents colony number. **Panel C** is a cell invasion through Matrigel assay. Invasion was quantified by crystal violet staining after 24 h. The top images represent control cell lines and the bottom images represent *CHD3* suppressed cell lines. **Panel D** is a bar graph of the results from the data from **Panel C**. The values are means ±SD (n=10) for three independent experiments performed in duplicate for at least 10 counted fields. $p<0.05$ (student t-test). The y-axis represents cells/ml and the x-axis is the cell lines used.

Figure 6-3

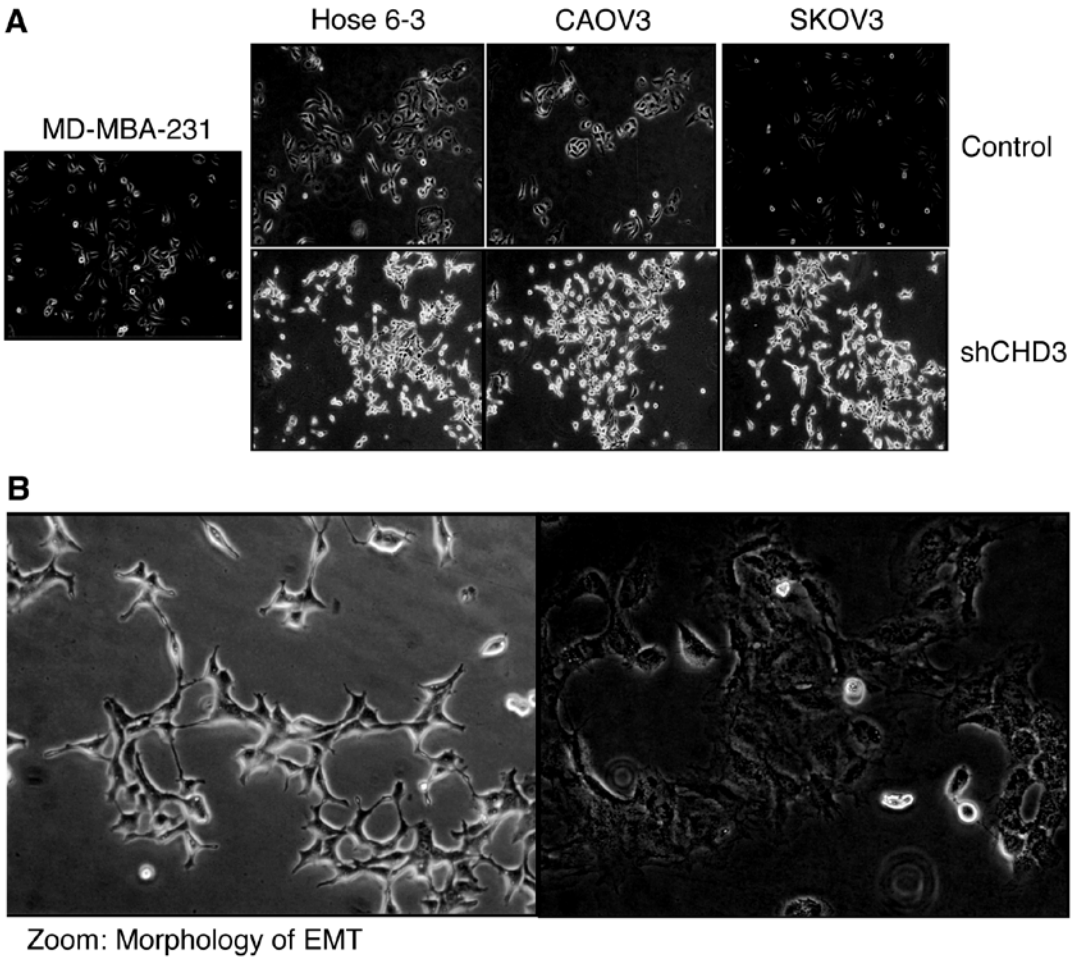


Figure 6-3 illustrates that cells with platinum-resistance induced by *CHD3* suppression are characterized by mesenchymal-like features. Images of phase contrast microscopy of cell line with and without *CHD3* suppression showing the cells undergone an EMT like morphology. The top panel is the control cell lines and the bottom panel is the sh*CHD3*

infected cell lines. MDA MB 232 is used as a reference control for a cell line that has undergone an EMT according to ATCC [272].

Figure 6-4

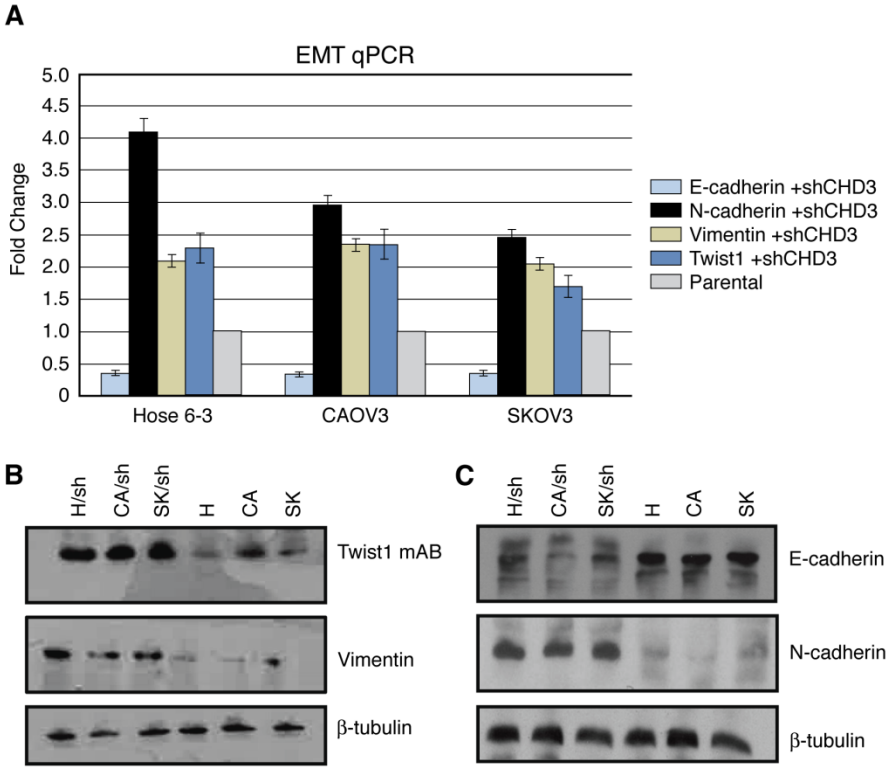


Figure 6-4 Illustrates the changes in EMT Associated Markers with the knock-down of *CHD3*. **Panel A** is a bar plot of RT-Q-PCR data analyzing the level of EMT associated markers in shRNA *CHD3* suppressed cells compared to non-target control cells. The markers analyzed by RT-Q-PCR shown are E-cadherin, N-cadherin, Vimentin and Twist. The Y axis is the normalized message level with the non-target reference shown at a level of 1 by definition. The bars on the X axis represent the cell lines used. Error bars illustrate standard deviation (n=3, $P<0.05$) **Panel B** is a western blot demonstrating that the protein levels correlate with the RT-Q-PCR data and change consistent with EMT. The top images are for the EMT associated markers and the bottom images the loading control β-tubulin. Cell lines are listed above the blots.

Figure 6-5

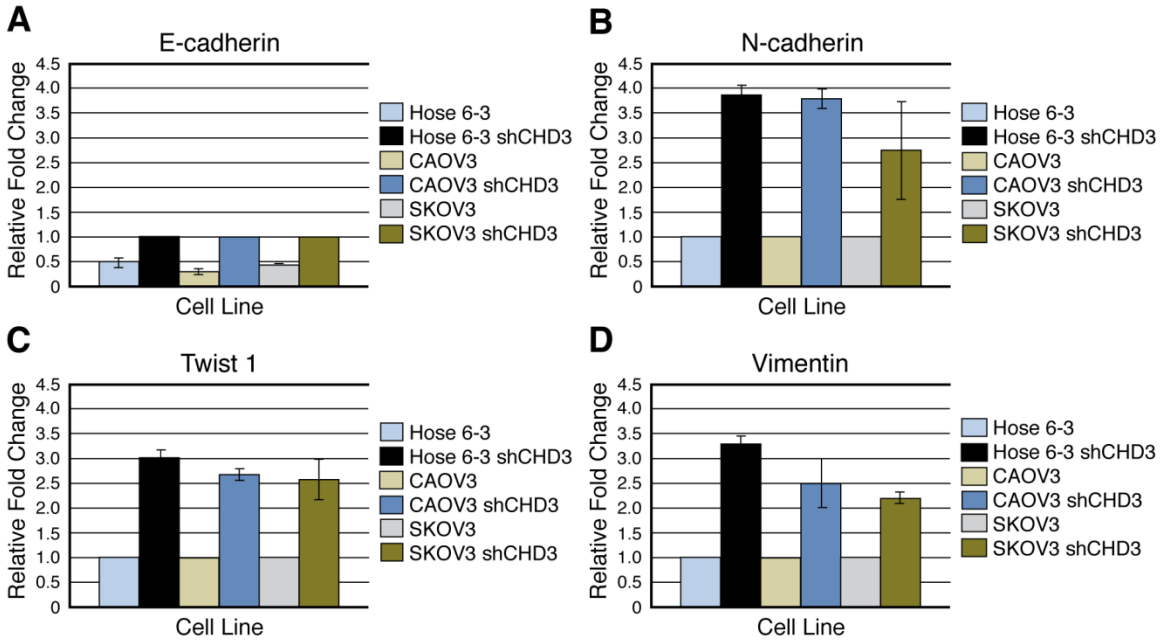


Figure 6-5 represents the western blot protein quantification. Quantification of lysates was done using ImageJ. Bar graphs represent the average of 3 independent experiments. Error bars represent standard deviations (n=3, P<0.06). The X axis is the cells lines, parental and shCHD3. The Y axis is the fold change of protein with the non target shown as 1 by definition of being the reference.

Table 6-1

Proteins that increase in abundance	Proteins that decrease in abundance	In vitro functional markers
N-cadherin	E-cadherin	Increased migration
Vimentin	Desmoplakin	Increased invasion
Fibronectin	Cytokeratin	Increased scattering
Snail1 (Snail)	Occludin	Elongation of cell shape
Snail2 (Slug)		Resistance to anoikis
FOXC2		
Sox10		
MMP-2		
MMP-3		
MMP-9		

Table 6.1 is a summary of the epithelial mesenchymal transition markers [273, 274]

Chapter VII: Conclusions and Perspectives

7.1 Summary

As drug resistance is one of the major barriers to the successful treatment of malignancies, investigation of the mechanisms of drug resistance and approaches to overcoming it has been widely performed in the past decades. Ovarian cancer is one of the most common cancers in women and the most lethal gynecological malignancy. The high mortality rate is attributable to the asymptomatic nature of the early stages of the disease, the lack of reliable screening tests, and the development of drug resistance [275].

Epigenetic inactivation of genes is crucial for control of normal cell growth and is a hallmark of cancer [1]. Epigenetic silencing occurring during tumor development has the potential to affect the chemo-sensitivity of tumor cells and how tumors respond to chemotherapy [109]. We have looked at the epigenome of ovarian cancer to study genes suppressed by DNA methylation and demonstrate that the chromatin remodeling protein, *CHD3*, is epigenetically regulated. We went on to show that the suppression of *CHD3* functionally increases the cells resistance to platinum drugs. Even when challenged with high doses of platinum based drugs, these cell lines survive and proliferate in vitro. To our knowledge, this is the first time this gene or any of the genes in this family have been implicated in sensitivity.

It is important to keep in mind that there are some limitations to this dissertation. First, we only looked at statistically significant genes that are suppressed by DNA methylation in resistant patients from the bioinformatics screen. It will be important to follow up with a similar screen that focuses on genes that become activated and/or

overexpressed in patients that are resistant to therapy. Also, because we generated such a large list of gene candidates, some of the less relevant genes may have been neglected in the screen. Therefore, influence of certain genes in the gene set list as well as normalization procedures may lead to inaccurate assessment of statistical significance in some instances. It may be informative to go back and repeat the functional screen for some of the less significant genes that were in the original gene set list. It is also highly likely that other epigenetic regulations are important in therapy response, such as acetylation. Furthermore, it would be fruitful to be able to correlate genetic mutations with therapy response, although to date this has been largely unsuccessful for ovarian cancer.

7.2 RNAi screen identifies potential genes involved in chemoresistance

Diversity and complexity are hallmarks of cancer genomes. Even tumors arising from the same cell type or tissue harbor a range of genetic lesions that facilitate their uncontrolled expansion and eventual metastasis. As a consequence, the behavior of individual tumors – how they progress and ultimately respond to therapy – is heterogeneous and unpredictable.

The goal of this study was to integrate cancer genomics, RNAi technology, and an in vitro tissue culture model to rapidly discover and validate cancer genes involved in therapy response. Our approach was based on the premise that the tumors take advantage of epigenetic mechanisms to become resistant to therapy and/or increase their ability to survive (**Figure 2-1 and 2-2**). In addition to identifying new genes and pathways

relevant to EOC and other tumor types, our study provides one approach for functionally annotating the cancer genome.

To identify epigenetically regulated genes directly associated with ovarian cancer chemotherapy resistance, a genome wide screen of DNA methylation was carried out on a set of ovarian tumors and those genes that segregated with resistance and sensitivity were identified. The most significant genes (**Table 2-1**) were then tested in an in-vitro culture system developed to assay the effect of platinum resistance. RNAi studies have infiltrated biology as a whole in many diverse areas of research, allowing for the study of the entire cancer genome. Therefore, to facilitate a more rapid approach in testing for genes functionally involved in platinum resistance, we employed an in vitro RNAi screen. First, we compiled a list of about 350 statistically significant genes that were methylated and transcriptionally represeed in resistant ovarian tumors when compared to sensitive tumors. Then, we ordered short hairpin RNAs from Open Biosystems and designed pools of about 50 genes. Then, we tested their ability to promote chemoresistance in a functional cell culture model. This screen was successful and allowed for the rapid identification of several genes that are predicted to play a functional role in therapy response. Future work in the lab will continue to look at the genes identified in the RNAi screen. The focus of this project is aimed at further validation of one gene, a chromodomain helicase DNA binding protein, CHD3 (**Figure 2-3**).

7.3 *CHD3* shRNAs causes resistance to both carboplatin and cisplatin

Following the results from the RNAi screen, we went on and did further functional experiments on *CHD3*. Dynamic regulation of chromatin structure is of fundamental importance for modulating genomic activities in eukaryotes [117]. Chromatin remodeling is essential for many processes including transcription. Therefore we predicted that this protein is playing an important role in therapy response.

Analysis of *CHD3* revealed that when the shRNA to *CHD3* is introduced into cells and these cells are challenged with carboplatin, they are able to escape and survive the treatment better than the parental control cells (**Figure 3-1**, **Panel A**). Furthermore, sh*CHD3* cells tend to grow slower than the controls (**Figure 3-2**). Moreover, as seen in **Figure 3-1 Panel B** these experiments were repeated using cisplatin and we obtained a similar result, which is expected since both drugs have a similar mechanism of action.

Several mechanisms that may be involved in drug resistance have been proposed, including a decrease in the accumulation of the drug, an increase in detoxification of the drug within the cell, and an increase in DNA repair activity (**Table 4-1**) [276]. The current regimen of chemotherapy for ovarian cancer consists of a combination of platinum and paclitaxel [8]. Although these compounds have improved treatment success rates over the past decade, the majority of patients experience a relapse, and in most patients, the disease persists. Proliferative activity is known to be related to chemoresponse [277]. Although resistance mechanisms have been extensively studied for platinum therapy, therapies to target resistance pathways have yet to be identified. We predict *CHD3* to be involved in chemoresistant pathways and that perhaps it may act a biomarker for chemoresistance. CHD3 is a chromatin remodeler and is thought to be

involved in transcriptional repression. Therefore, it is possible that CHD3 regulates genes involved in resistant pathways, such as DNA repair genes and apoptosis genes.

7.4 Role of *CHD3* in platinum uptake/efflux

There are a number of different basic mechanisms for a cell to become chemo resistant; first the uptake can be decreased [278], two the efflux of the drug can be increased [279], three another mechanism that allows the cells to tolerate the chemotherapy, probably by altering a target in the cell. We tested the first two alternatives and found no change in influx or the efflux when comparing cells with *CHD3* suppression to parent cells (**Table 4-1** and **Table 4-2**). Therefore we concluded that the resistance was a result of some other mechanism independent of the amount of Carboplatin getting into the cells. Several genes such as EGFR and k-ras have been implicated as having an important role in many cancers, where they act to enhance chemoresistance and proliferation together [280, 281]. Somewhat surprisingly, cells with repressed *CHD3*, exhibit a decrease in cellular proliferation (**Figure 3-2**). We propose that by growing slower the cells incorporate less platinum reducing the level of DNA adducts, thus being more resistant. This possible mechanism has been reported in several laboratories for ovarian tumors and tumor cell lines [197, 198, 282]. We tested this by isolating the DNA from the cells and measuring the platinum in the DNA and found that the level of platinum in the cells was indeed lower. While other mechanisms are possible we concluded that it is likely that the cells with *CHD3* suppression grow slower and incorporate less platinum into the DNA, thereby becoming more resistant. Therefore we

have concluded that the cells have developed a mechanism to better tolerate the presence of Carboplatin. Because *CHD3* is thought to be involved in transcriptional repression, we predict it to play a role in several pathways thought to be involved in chemotherapy tolerance. For example, *CHD3* may target genes such as excision repair cross-complementing rodent repair deficiency, complementation group 1 (*ERCC1*) and xeroderma pigmentosum group B (*XPB*), involved in the nucleotide excision repair pathway in epithelial ovarian cancer tumors. Increased expression of these genes is thought increased repair activity and lead to chemoresistance in clear cell carcinoma [51]. It will be interesting to follow up with this in the future.

7.5 *CHD3* associated response to apoptosis and senescence

Modulation of apoptosis may influence resistance to chemotherapy and therefore affect the outcome of cancer treatment. It is well established that platinum induces DNA damage through formation of inter- and intra-strand cross-linkages and activation of p53-dependent apoptotic pathways. Alterations in platinum cellular retention or excretion, DNA repair mechanism, or apoptotic machinery have been considered important etiological factors in chemoresistance. In ovarian cancer p53 status is a strong predictor of response to platinum-based chemotherapy. Patients whose tumors have p53 mutations experience a lower chance of achieving a complete response following platinum-based regimens when compared to patients without p53 mutations [283]. Another marker for apoptosis is γH2AX. Chromosomal DNA wraps around histone proteins to form a complex scaffold called chromatin [284]. The reorganization of these proteins following

DNA damage is crucial for repairing the damage, and so maintaining genomic integrity and reducing the likelihood of cell death or cancer. γH2AX is one such histone modification follows DNA double-strand breaks (DSBs) and involves phosphorylation by the enzyme ATM of serine residue 139, which is located in the carboxy terminal tail of the histone variant H2AX. Caspase 3 is another marker used for apoptosis that becomes proteolytically cleaved when cells are triggered to undergo apoptosis [222]. Multiple apoptotic signals can activate Caspase-3, including chemotherapy agents and UV-radiation [285].

To determine if there was a difference in well characterized p53 dependent apoptotic mechanisms in response to loss of *CHD3* we tested this by assaying two different markers of apoptosis, caspase 3 production and the accumulation of the γH2AX phosphorylation. For both marker assays, detection of caspase 3 and γH2AX phosphorylation as well as the production of classical apoptosis associated DNA smear we obtained similar results. In the immortalized HOSE cells without the shRNA to *CHD3* upon treatment with platinum drug, apoptosis was induced as measured by the production of large amounts of caspase 3 or γH2AX phosphorylation. However, with the addition of the shRNA to *CHD3*, platinum no longer caused extensive production of caspase 3 or γH2AX phosphorylation (**Figure 5-1 Panel A-C**).

Additionally, because platinum drugs work by crosslinking DNA and thereby introducing DNA DSBs to induce apoptosis, we therefore examined DNA fragmentation on an ethidium bromide gel. Degradation of nuclear DNA into nucleosomal units is one of the hallmarks of apoptotic cell death. It occurs in response to various apoptotic stimuli in a wide variety of cell types producing cleavage of chromatin DNA into internucleosomal

fragments of 180 bp and multiples thereof. As seen in **Figure 5-1 Panel D** our results indicate that DNA fragmentation occurs in our chemosensitive population of cells and not in those expressing the shRNA to *CHD3*. As expected, there is some amount of DNA laddering in tumor cells without shRNA to *CHD3* but it is less produced from cells which have the *CHD3* shRNA. This suggests that the cells treated with platinum chemotherapy do undergo apoptosis but do so at a lower rate in cells with the *CHD3* shRNA. From these results we can see that the decreased number of cells when shRNA to the *CHD3* gene is introduced is not caused by an increase in apoptosis. Therefore, we predict that *CHD3* is playing a functional role in chemoresistance by directly inhibiting some effector(s) of apoptosis. We speculate that there is a lack of coordination between the cell cycle and DNA repair in the resistant cell lines allowing proliferation, albeit slow, in the presence of DNA damage which has created an increase in genomic instability.

In the future we aim to study the role of more players in apoptosis pathway both p53 dependent and p53 independent. In addition, it will be important to examine the progression of these cells through the cell cycle to see if that is being altered to evade apoptosis. We predict *CHD3* may regulate genes involved in apoptosis that specifically allow the cell to escape cell death such as upregulating the antiapototic protein Bcl-2 [286]. Aberrant expression of Bcl-2 family proteins is mostly regulated at the transcriptional level [287] and therefore it is plausible that *CHD3* may play a role in this regulation or of other antiapoptotic genes. Analogous to that prediction, CHD3 may decrease the transcription of proapoptotic genes.

A possible explanation for the observed decrease in total number of cells in culture with the suppression of *CHD3* is induction of senescence. To test this we assayed

for senescence associated beta galactosidase activity. We obtained similar results to what we found for the analysis of apoptosis (**Figure 5-2**). The only cell line that showed senescence after treatment with Carboplatin was the HOSE cells without the introduction of the *CHD3* shRNA. This might be due to the fact that these cells are immortalized with HPV E6/7 ORF and that *CHD3* may trigger a senescence checkpoint in non-transformed cells, but this rarely occurs. The other cell lines had comparable levels of senescence. These data show us that in tumor cells the loss of *CHD3* does not induce senescence considerable and that some other mechanism accounts for platinum resistance in these cell lines.

Since there was not increased senescence and the contrary, there was actually a decrease in apoptosis we hypothesized that *CHD3* suppression alters cell cycle progression. Cell proliferation depends on the ability of the cell to successfully pass through the G1, S, G2, and M phases of the cell cycle. Cytotoxic drugs are primarily effective against proliferating cells; therefore, quiescent cells show a degree of drug resistance relative to cycling cells [288, 289]. Specifically, platinum drugs are thought to be effective by cross-linking DNA between two adjacent guanine residues on the same strand of DNA [198]. We propose that by growing slower the cells incorporate less platinum reducing the level of DNA adducts, thus being more resistant. Therefore, we predict that platinum resistant cells that are transcriptionally silenced for *CHD3* progress slowly through the cell cycle and avoid uptake of platinum drugs. Specifically, cells would arrest in the S phase of cell cycle, as this is where platinum would induce the most damage. Currently we are testing this hypothesis in the lab. Preliminary results reveal that 24 hours after nocodazole arrest release the cells with the shRNA to *CHD3*

accumulated more G2/M portion of the cell cycle (data not shown**).** It appears that suppression of *CHD3* does cause a decrease in the cell cycle which accounts for the decreased number of cells observed in the shRNA containing cells. This experiment is currently being repeated for further validation. It is possible that *CHD3* regulates several genes involved in cell cycle progression and check point. Furthermore, arrest in G2/M is needed for establishment of heritably repressed chromatin, and because *CHD3* is a chromatin remodeling factor, it may play a role in this regulation [290].

7.6 *CHD3* associated platinum resistance may be caused by EMT

Since there was not a change in uptake or efflux of carboplatin, we hypothesized that some other mechanism was responsible for chemoresistance in the cells silenced for CHD3. We noticed that the cells transcriptionally silenced for CHD3 had change in morphology that seemed consistent with epithelial-mesenchymal transition (EMT) and therefore performed several assays to assess platinum resistant-epithelial ovarian carcinoma (EOC) cells for cellular morphology, motility, and molecular changes consistent with EMT. EMT is a process initially observed in embryonic development in which cells lose epithelial characteristics and gain mesenchymal properties to increase motility and invasion [239]. Previous research suggests that EMT is also important in tumor progression and metastasis [239, 291], as well as chemoresistance [246].

In this study, we observed that platinum-resistant EOC cells exhibit an altered phenotype whereby cells disperse, develop pseudopodia, and assume a spindle shape, properties associated with the EMT phenotype. Based on the above observations, we

hypothesized that platinum resistance leads to an EMT phenotype, including its characteristic molecular alterations. To test this hypothesis, we assessed EOC cells for gross morphologic and molecular changes consistent with EMT. We showed in this series of experiments that acquisition of platinum resistance by EOC cells with the shRNA to *CHD3* leads to morphologic and molecular alterations consistent with a change to a mesenchymal-like phenotype (**Figure 6-3**). To examine EMT molecular changes, we tested for EMT related markers. As outlined in **Figure 6-4** these include mesenchymal specific markers such as Vimentin and N-cadherin [240], epithelial specific markers such as E-cadherin [241] and the transcription factor TWIST [242]. We concluded that the gross morphological changes associated with EMT were consistent with molecular changes in cells transcriptionally silenced for *CHD3*.

Recent research has implicated EMT in cancer progression by noting that epithelial-derived tumor cells can switch their phenotype to a more primitive mesenchymal phenotype that facilitates motility and invasion [239]. We believe that our finding that EOC cells with shRNA to *CHD3* undergo epithelial to mesenchymal or mesenchymal-like transition reflects an important process by which cancer cells may potentially acquire chemoresistance. We hypothesize that EOC cells with shRNA to *CHD3* may switch their molecular machinery from a proliferative, epithelial phenotype to a more invasive and migratory phenotype. Because proliferation is required for platinum-induced chemosensitivity, the decrease in proliferation of EOC cells with shRNA to *CHD3* may be one means whereby resistant cells can escape the effects of chemotherapy. It may do so by directly affecting the transcription of genes involved in cell cycle progression (*e.g.* cyclin dependent kinases, Rb) and/or EMT. Furthermore, EMT may

also be a mechanism of escape from apoptosis [265]. This may be one explanation for the decrease in apoptosis that we noted in the CHD3 suppressed cells. Therefore, the acquisition of mesenchymal phenotypes engenders tumor cells with a multifaceted capacity to proliferate, migrate, and avoid cell death and permanent arrest, as well as protection from extracellular signals and drug effect activities.

Interestingly, recent studies have shown that gemcitabine-resistant cells acquired EMT phenotype, which is reminiscent of cancer stem-like cells [247]. This phenotype was associated with spindle-shaped morphology and enhanced pseudopodia formation. The gemcitabine-resistant cells also showed decreased expression of the epithelial adhesion molecule E-cadherin and an increase in the expression of mesenchymal markers such as Vimentin [247]. This was similar to what we see in cell lines transcriptionally repressed for *CHD3,* suggesting a possible role for *CHD3* in stemness. Given that the chip-chip data revealed that *CHD3* binds to the promoter of *FOXD3* which is required for the maintenance of pluripotent cells, this further provides evidence for a possible role of *CHD3* in stem-cell like maintenance [190]. Furthermore, Twist1 may also be an important regulator of "stemness" in EOC cells [292]. Taken together, this data suggests that EOC cells, by going through an EMT, acquire stemness characteristics qualifying them to acquire chemoresistance and recent reports provide evidence that this may be done by overcoming p53-mediated apoptosis [293], which is consistent with our data. In the future it will be interesting to determine if the hypothesis of CHD3 suppression induces a cancer stem cell phenotype.

In conclusion, this is, to our knowledge, the first description of chemoresistance-induced EMT, and we postulate that EMT induced by acquisition of platinum resistance

could be a possible survival mechanism for chemoresistant EOC cells with shRNA to *CHD3*. The present study reveals the molecular mechanisms of resistance acquired by the epigenetic repression of *CHD3* through EMT transcriptional programs. If our hypothesis is true, blocking or reversing EMT changes may cause chemoresistant cells to revert to chemosensitive cells. Additionally, this may represent a mechanism whereby other cancer cells generate chemoresistance to a multitude of drugs, providing a role for *CHD3* as a biomarker for chemoresistance. We predict this to be an exciting area of research in the future, and hope that it will benefit EOC patients with acquired chemoresistance.

7.7 Future outlook

Previous results have indicated that EMT-associated genes including *Twist*, play a role in EMT associated cellular resistance to paclitaxel in human ovarian cancer [294]. Our results indicate that when *CHD3* is repressed, the cellular morphology change to a EMT like state and we found agreement on the state of markers of EMT by transcript and protein level. Therefore we have concluded that loss of *CHD3* initiates some path to the transition from epithelial to a mesenchymal state. *CHD3* is a subunit of a large chromatin remodeling complex, NuRD. Interestingly other members of the NuRD complex are also associated with EMT including HDAC1, HDAC2, MTA1, rbAP46 [295-298]). As discussed above EMT and many regulators of EMT have been shown to increase resistance to a wide variety of chemotherapies. It is understandable that alteration of a chromatin modifier such as *CHD3* can induce EMT and result in chemotherapy

resistance. It is not surprising that HDAC inhibitors are clinically effective at inhibiting tumor growth. In fact the treatment of tumor cells with HDAC inhibitors block EMT or even causes the reversion of EMT in some cases [294, 299, 300].

We have shown that the suppression of *CHD3* in cells increases resistance to platinum based drugs, by slowing the growth of the cells, while still increasing the invasive and metastatic properties. Generally the increase of HDAC activity is associated with increased EMT [297, 300], suggesting that *CHD3* has some role in either suppressing or more likely specifically regulating the target selection for the HDAC complex. [114]. There are many questions left unanswered as to how *CHD3* regulates resistance to platinum based drugs. How does suppression of *CHD3* link to EMT? With suppression of *CHD3* is there an effect on the expression of target genes of the HDAC complex and are these target genes the primary or secondary targets involved in EMT. The specific role of *CHD3* in the NuRD complex is not fully understood however it may focus around histone deacetylase activity, which usually associates with transcriptional repression [301]. *CHD3* may participate in transcriptional repression by assisting repressors in gaining access to chromatin. We find suppression of *CHD3* causing increased resistance to chemo treatment but targeting gain of function as a clinical treatment for cancer is very difficult. In addition the same effect on platinum resistance may occur with the suppression of the family member CHD4 which we also see methylated and are now analyzing further. For this reason it may be more realistic to target the HDAC complex by targeting the HDAC itself, by using HDAC inhibitors. This may revert EMT in ovarian cancer cells and induce sensitivity to platinum based drugs. Overall, our work has identified *CHD3* to be an important player in chemoresistance in

EOC. Therefore, we predict that patients with *CHD3* suppressed might be candidates for HDAC inhibitor cancer therapy. Furthermore, it might also explain why original trials using HDAC inhibitors were unsuccessful in that there may have been a wrong selection of patients for the HDAC treatment. We predict *CHD3* to be a biomarker for patients who might respond well to HDAC inhibitor treatment. If this hypothesis is correct, treatment of some patients with combination therapy of HDAC inhibitor and platinum based drugs may have a more favorable outcome for survival. These directions will be the study of future work. By carrying out this project we have taken critical steps into understanding the mechanism that promoter methylation plays in therapy response of ovarian cancer.

References

1. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.
2. Weigelt, B., J.L. Peterse, and L.J. van 't Veer, *Breast cancer metastasis: markers and models.* Nat Rev Cancer, 2005. **5**(8): p. 591-602.
3. Fodale, V., et al., *Mechanism of cell adaptation: when and how do cancer cells develop chemoresistance?* Cancer J. **17**(2): p. 89-95.
4. Lonning, P.E., *Molecular basis for therapy resistance.* Mol Oncol. **4**(3): p. 284-300.
5. Simstein, R., et al., *Apoptosis, chemoresistance, and breast cancer: insights from the MCF-7 cell model system.* Exp Biol Med (Maywood), 2003. **228**(9): p. 995-1003.
6. Gu, B., et al., *Organ-selective chemoresistance in metastasis from human breast cancer cells: inhibition of apoptosis, genetic variability and microenvironment at the metastatic focus.* Carcinogenesis, 2004. **25**(12): p. 2293-301.
7. Bergman, P.J. and D. Harris, *Radioresistance, chemoresistance, and apoptosis resistance. The past, present, and future.* Vet Clin North Am Small Anim Pract, 1997. **27**(1): p. 47-57.
8. McCluskey, L.L. and L. Dubeau, *Biology of ovarian cancer.* Curr Opin Oncol, 1997. **9**(5): p. 465-70.
9. Werness, B.A. and G.H. Eltabbakh, *Familial ovarian cancer and early ovarian cancer: biologic, pathologic, and clinical features.* Int J Gynecol Pathol, 2001. **20**(1): p. 48-63.
10. Tiersten, A.D., et al., *Phase II evaluation of neoadjuvant chemotherapy and debulking followed by intraperitoneal chemotherapy in women with stage III and IV epithelial ovarian, fallopian tube or primary peritoneal cancer: Southwest Oncology Group Study S0009.* Gynecol Oncol, 2009. **112**(3): p. 444-9.
11. Aichinger, E., et al., *CHD3 proteins and polycomb group proteins antagonistically determine cell identity in Arabidopsis.* PLoS Genet, 2009. **5**(8): p. e1000605.
12. Tong, J.K., et al., *Chromatin deacetylation by an ATP-dependent nucleosome remodelling complex.* Nature, 1998. **395**(6705): p. 917-21.
13. Jemal, A., et al., *Cancer statistics, 2010.* CA Cancer J Clin. **60**(5): p. 277-300.
14. Schwartz, P.E., *Current diagnosis and treatment modalities for ovarian cancer.* Cancer Treat Res, 2002. **107**: p. 99-118.
15. Goff, B.A., et al., *Frequency of symptoms of ovarian cancer in women presenting to primary care clinics.* JAMA, 2004. **291**(22): p. 2705-12.
16. Agarwal, R. and S.B. Kaye, *Ovarian cancer: strategies for overcoming resistance to chemotherapy.* Nat Rev Cancer, 2003. **3**(7): p. 502-16.
17. Auersperg, N., et al., *Ovarian surface epithelium: biology, endocrinology, and pathology.* Endocr Rev, 2001. **22**(2): p. 255-88.
18. Marcickiewicz, J. and M. Brannstrom, *Fertility preserving surgical treatment of borderline ovarian tumour: long-term consequence for fertility and recurrence.* Acta Obstet Gynecol Scand, 2006. **85**(12): p. 1496-500.

19. Sorbe, B., et al., *Treatment of primary advanced and recurrent endometrial carcinoma with a combination of carboplatin and paclitaxel-long-term follow-up.* Int J Gynecol Cancer, 2008. **18**(4): p. 803-8.
20. Kosary, C.L., *FIGO stage, histology, histologic grade, age and race as prognostic factors in determining survival for cancers of the female gynecological system: an analysis of 1973-87 SEER cases of cancers of the endometrium, cervix, ovary, vulva, and vagina.* Semin Surg Oncol, 1994. **10**(1): p. 31-46.
21. Bellacosa, A., et al., *Altered gene expression in morphologically normal epithelial cells from heterozygous carriers of BRCA1 or BRCA2 mutations.* Cancer Prev Res (Phila). **3**(1): p. 48-61.
22. D'Arcy, T.J., et al., *Ovarian cancer detected non-invasively by contrast-enhanced power Doppler ultrasound.* BJOG, 2004. **111**(6): p. 619-22.
23. Baker, V.V., et al., *c-myc amplification in ovarian cancer.* Gynecol Oncol, 1990. **38**(3): p. 340-2.
24. Chou, J.L., et al., *TGF-beta: friend or foe? The role of TGF-beta/SMAD signaling in epigenetic silencing of ovarian cancer and its implication in epigenetic therapy.* Expert Opin Ther Targets. **14**(11): p. 1213-23.
25. Cleaver, J.E., et al., *Increased ultraviolet sensitivity and chromosomal instability related to P53 function in the xeroderma pigmentosum variant.* Cancer Res, 1999. **59**(5): p. 1102-8.
26. Luo, R.Z., et al., *ARHI is a Ras-related small G-protein with a novel N-terminal extension that inhibits growth of ovarian and breast cancers.* Oncogene, 2003. **22**(19): p. 2897-909.
27. Tagliaferri, P., et al., *BRCA1/2 genetic background-based therapeutic tailoring of human ovarian cancer: hope or reality?* J Ovarian Res, 2009. **2**: p. 14.
28. Jongsma, A.P., et al., *Molecular evidence for putative tumour suppressor genes on chromosome 13q specific to BRCA1 related ovarian and fallopian tube cancer.* Mol Pathol, 2002. **55**(5): p. 305-9.
29. Dai, Z., Y. Huang, and W. Sadee, *Growth factor signaling and resistance to cancer chemotherapy.* Curr Top Med Chem, 2004. **4**(13): p. 1347-56.
30. Papac, R.J., *Origins of cancer therapy.* Yale J Biol Med, 2001. **74**(6): p. 391-8.
31. Umezu, T., et al., *Taxol resistance among the different histological subtypes of ovarian cancer may be associated with the expression of class III beta-tubulin.* Int J Gynecol Pathol, 2008. **27**(2): p. 207-12.
32. Dierickx, K.M., et al., *Improving the spectrophotometric determination of the alkylating activity of anticancer agents: a new insight into the mechanism of the NBP method.* Talanta, 2009. **77**(4): p. 1370-5.
33. Shridhar, V., et al., *Loss of expression of a new member of the DNAJ protein family confers resistance to chemotherapeutic agents used in the treatment of ovarian cancer.* Cancer Res, 2001. **61**(10): p. 4258-65.
34. Jazaeri, A.A., et al., *Gene expression profiles associated with response to chemotherapy in epithelial ovarian cancers.* Clin Cancer Res, 2005. **11**(17): p. 6300-10.
35. Spentzos, D., et al., *IGF axis gene expression patterns are prognostic of survival in epithelial ovarian cancer.* Endocr Relat Cancer, 2007. **14**(3): p. 781-90.

36. Bild, A.H., A. Potti, and J.R. Nevins, *Linking oncogenic pathways with therapeutic opportunities.* Nat Rev Cancer, 2006. **6**(9): p. 735-41.
37. Etemadmoghadam, D., et al., *Integrated genome-wide DNA copy number and expression analysis identifies distinct mechanisms of primary chemoresistance in ovarian carcinomas.* Clin Cancer Res, 2009. **15**(4): p. 1417-27.
38. Borst, P., et al., *A family of drug transporters: the multidrug resistance-associated proteins.* J Natl Cancer Inst, 2000. **92**(16): p. 1295-302.
39. Kigawa, J., et al., *Gamma-glutamyl cysteine synthetase up-regulates glutathione and multidrug resistance-associated protein in patients with chemoresistant epithelial ovarian cancer.* Clin Cancer Res, 1998. **4**(7): p. 1737-41.
40. Kamazawa, S., et al., *Multidrug resistance gene-1 is a useful predictor of Paclitaxel-based chemotherapy for patients with ovarian cancer.* Gynecol Oncol, 2002. **86**(2): p. 171-6.
41. Godwin, A.K., et al., *Growth regulation of ovarian cancer.* Hematol Oncol Clin North Am, 1992. **6**(4): p. 829-41.
42. Friesen, C., Y. Kiess, and K.M. Debatin, *A critical role of glutathione in determining apoptosis sensitivity and resistance in leukemia cells.* Cell Death Differ, 2004. **11 Suppl 1**: p. S73-85.
43. Ghazal-Aswad, S., A.H. Calvert, and D.R. Newell, *A single-sample assay for the estimation of the area under the free carboplatin plasma concentration versus time curve.* Cancer Chemother Pharmacol, 1996. **37**(5): p. 429-34.
44. Wrigley, E.C., et al., *Glutathione-S-transferase activity and isoenzyme levels measured by two methods in ovarian cancer, and their value as markers of disease outcome.* Br J Cancer, 1996. **73**(6): p. 763-9.
45. Tanner, B., et al., *Glutathione, glutathione S-transferase alpha and pi, and aldehyde dehydrogenase content in relationship to drug resistance in ovarian cancer.* Gynecol Oncol, 1997. **65**(1): p. 54-62.
46. Koberle, B., et al., *DNA repair capacity and cisplatin sensitivity of human testis tumour cells.* Int J Cancer, 1997. **70**(5): p. 551-5.
47. Zeng-Rong, N., et al., *Elevated DNA repair capacity is associated with intrinsic resistance of lung cancer to chemotherapy.* Cancer Res, 1995. **55**(21): p. 4760-4.
48. Johnson, S.W., et al., *Role of platinum-DNA adduct formation and removal in cisplatin resistance in human ovarian cancer cell lines.* Biochem Pharmacol, 1994. **47**(4): p. 689-97.
49. Yen, L., et al., *Enhanced host cell reactivation capacity and expression of DNA repair genes in human breast cancer cells resistant to bi-functional alkylating agents.* Mutat Res, 1995. **337**(3): p. 179-89.
50. Ali-Osman, F., A. Rairkar, and P. Young, *Formation and repair of 1,3-bis-(2-chloroethyl)-1-nitrosourea and cisplatin induced total genomic DNA interstrand crosslinks in human glioma cells.* Cancer Biochem Biophys, 1995. **14**(4): p. 231-41.
51. Reed, E., et al., *Clear cell tumors have higher mRNA levels of ERCC1 and XPB than other histological types of epithelial ovarian cancer.* Clin Cancer Res, 2003. **9**(14): p. 5299-305.
52. Claij, N. and H. te Riele, *Microsatellite instability in human cancer: a prognostic marker for chemotherapy?* Exp Cell Res, 1999. **246**(1): p. 1-10.

53. Aebi, S., et al., *A phase II/pharmacokinetic trial of high-dose progesterone in combination with paclitaxel.* Cancer Chemother Pharmacol, 1999. **44**(3): p. 259-65.
54. Fink, D., et al., *The role of DNA mismatch repair in platinum drug resistance.* Cancer Res, 1996. **56**(21): p. 4881-6.
55. Shigemasa, K., et al., *Cyclin D1 overexpression and p53 mutation status in epithelial ovarian cancer.* J Soc Gynecol Investig, 1999. **6**(2): p. 102-8.
56. McDonald, E.R., 3rd, et al., *Repair Defect in p21 WAF1/CIP1 -/- human cancer cells.* Cancer Res, 1996. **56**(10): p. 2250-5.
57. Dalerba, P. and M.F. Clarke, *Cancer stem cells and tumor metastasis: first steps into uncharted territory.* Cell Stem Cell, 2007. **1**(3): p. 241-2.
58. Sanchez-Garcia, I., C. Vicente-Duenas, and C. Cobaleda, *The theoretical basis of cancer-stem-cell-based therapeutics of cancer: can it be put into practice?* Bioessays, 2007. **29**(12): p. 1269-80.
59. Miyashita, T. and J.C. Reed, *Bcl-2 oncoprotein blocks chemotherapy-induced apoptosis in a human leukemia cell line.* Blood, 1993. **81**(1): p. 151-7.
60. Fraser, M., et al., *p53 is a determinant of X-linked inhibitor of apoptosis protein/Akt-mediated chemoresistance in human ovarian cancer cells.* Cancer Res, 2003. **63**(21): p. 7081-8.
61. Zaffaroni, N., et al., *Expression of the anti-apoptotic gene survivin correlates with taxol resistance in human ovarian cancer.* Cell Mol Life Sci, 2002. **59**(8): p. 1406-12.
62. Servidei, T., et al., *The novel trinuclear platinum complex BBR3464 induces a cellular response different from cisplatin.* Eur J Cancer, 2001. **37**(7): p. 930-8.
63. Li, J., et al., *Human ovarian cancer and cisplatin resistance: possible role of inhibitor of apoptosis proteins.* Endocrinology, 2001. **142**(1): p. 370-80.
64. Li, Y., D. Dowbenko, and L.A. Lasky, *AKT/PKB phosphorylation of p21Cip/WAF1 enhances protein stability of p21Cip/WAF1 and promotes cell survival.* J Biol Chem, 2002. **277**(13): p. 11352-61.
65. Verhagen, A.M., E.J. Coulson, and D.L. Vaux, *Inhibitor of apoptosis proteins and their relatives: IAPs and other BIRPs.* Genome Biol, 2001. **2**(7): p. REVIEWS3009.
66. Galmarini, C.M., et al., *Drug resistance associated with loss of p53 involves extensive alterations in microtubule composition and dynamics.* Br J Cancer, 2003. **88**(11): p. 1793-9.
67. Hugo, H., et al., *Epithelial--mesenchymal and mesenchymal--epithelial transitions in carcinoma progression.* J Cell Physiol, 2007. **213**(2): p. 374-83.
68. Rosano, L., et al., *Acquisition of chemoresistance and EMT phenotype is linked with activation of the endothelin A receptor pathway in ovarian carcinoma cells.* Clin Cancer Res. **17**(8): p. 2350-60.
69. Hiscox, S., et al., *Elevated Src activity promotes cellular invasion and motility in tamoxifen resistant breast cancer cells.* Breast Cancer Res Treat, 2006. **97**(3): p. 263-74.
70. Yao, H., et al., *RhoC GTPase is required for PC-3 prostate cancer cell invasion but not motility.* Oncogene, 2006. **25**(16): p. 2285-96.

71. Tiersten, A.D., et al., *Chemotherapy resistance as a predictor of progression-free survival in ovarian cancer patients treated with neoadjuvant chemotherapy and surgical cytoreduction followed by intraperitoneal chemotherapy: a Southwest Oncology Group Study.* Oncology, 2009. **77**(6): p. 395-9.
72. Winter-Roach, B.A., H.C. Kitchener, and H.O. Dickinson, *Adjuvant (post-surgery) chemotherapy for early stage epithelial ovarian cancer.* Cochrane Database Syst Rev, 2009(1): p. CD004706.
73. Thomadaki, H. and A. Scorilas, *Molecular profile of breast versus ovarian cancer cells in response to treatment with the anticancer drugs cisplatin, carboplatin, doxorubicin, etoposide and taxol.* Biol Chem, 2008. **389**(11): p. 1427-34.
74. Chu, G., *Cellular responses to cisplatin. The roles of DNA-binding proteins and DNA repair.* J Biol Chem, 1994. **269**(2): p. 787-90.
75. Rose, P.G., et al., *Second-line therapy with paclitaxel and carboplatin for recurrent disease following first-line therapy with paclitaxel and platinum in ovarian or peritoneal carcinoma.* J Clin Oncol, 1998. **16**(4): p. 1494-7.
76. Barry, M.A., C.A. Behnke, and A. Eastman, *Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia.* Biochem Pharmacol, 1990. **40**(10): p. 2353-62.
77. Blommaert, F.A. and C.P. Saris, *Detection of platinum-DNA adducts by 32P-postlabelling.* Nucleic Acids Res, 1995. **23**(8): p. 1300-6.
78. Rodenburg, C.J. and F.J. Cleton, *Chemotherapy in advanced ovarian cancer.* J Cancer Res Clin Oncol, 1984. **107**(2): p. 99-105.
79. Wood, L.D., et al., *The genomic landscapes of human breast and colorectal cancers.* Science, 2007. **318**(5853): p. 1108-13.
80. Nakayama, N., et al., *Gene amplification CCNE1 is related to poor survival and potential therapeutic target in ovarian cancer.* Cancer. **116**(11): p. 2621-34.
81. Budd, M.E. and J.L. Campbell, *The pattern of sensitivity of yeast dna2 mutants to DNA damaging agents suggests a role in DSB and postreplication repair pathways.* Mutat Res, 2000. **459**(3): p. 173-86.
82. Tran, H.M., et al., *Mutant Nbs1 enhances cisplatin-induced DNA damage and cytotoxicity in head and neck cancer.* Otolaryngol Head Neck Surg, 2004. **131**(4): p. 477-84.
83. Wang, W. and W.D. Figg, *Secondary BRCA1 and BRCA2 alterations and acquired chemoresistance.* Cancer Biol Ther, 2008. **7**(7): p. 1004-5.
84. Moynahan, M.E., et al., *Brca1 controls homology-directed DNA repair.* Mol Cell, 1999. **4**(4): p. 511-8.
85. Swisher, E.M., et al., *Secondary BRCA1 mutations in BRCA1-mutated ovarian carcinomas with platinum resistance.* Cancer Res, 2008. **68**(8): p. 2581-6.
86. Geiman, T.M. and K.D. Robertson, *Chromatin remodeling, histone modifications, and DNA methylation-how does it all fit together?* J Cell Biochem, 2002. **87**(2): p. 117-25.
87. Jones, P.A. and S.B. Baylin, *The fundamental role of epigenetic events in cancer.* Nat Rev Genet, 2002. **3**(6): p. 415-28.
88. Galm, O., et al., *SOCS-1, a negative regulator of cytokine signaling, is frequently silenced by methylation in multiple myeloma.* Blood, 2003. **101**(7): p. 2784-8.

89. Xing, J., et al., *Alu element mutation spectra: molecular clocks and the effect of DNA methylation.* J Mol Biol, 2004. **344**(3): p. 675-82.
90. Terasawa, K., et al., *Epigenetic inactivation of TMS1/ASC in ovarian cancer.* Clin Cancer Res, 2004. **10**(6): p. 2000-6.
91. Ding, Y.B., et al., *Association of VCAM-1 overexpression with oncogenesis, tumor angiogenesis and metastasis of gastric carcinoma.* World J Gastroenterol, 2003. **9**(7): p. 1409-14.
92. Seidl, S., et al., *DNA-methylation analysis identifies the E-cadherin gene as a potential marker of disease progression in patients with monoclonal gammopathies.* Cancer, 2004. **100**(12): p. 2598-606.
93. Chiang, J.W., et al., *BRCA1 promoter methylation predicts adverse ovarian cancer prognosis.* Gynecol Oncol, 2006. **101**(3): p. 403-10.
94. Murawska, M., et al., *dCHD3, a novel ATP-dependent chromatin remodeler associated with sites of active transcription.* Mol Cell Biol, 2008. **28**(8): p. 2745-57.
95. de Vogel, S., et al., *MGMT and MLH1 promoter methylation versus APC, KRAS and BRAF gene mutations in colorectal cancer: indications for distinct pathways and sequence of events.* Ann Oncol, 2009. **20**(7): p. 1216-22.
96. Salam, I., et al., *Aberrant promoter methylation and reduced expression of p16 gene in esophageal squamous cell carcinoma from Kashmir valley: a high-risk area.* Mol Cell Biochem, 2009. **332**(1-2): p. 51-8.
97. Maradeo, M.E. and P. Cairns, *Translational application of epigenetic alterations: Ovarian cancer as a model.* FEBS Lett.
98. Baldwin, R.L., et al., *BRCA1 promoter region hypermethylation in ovarian carcinoma: a population-based study.* Cancer Res, 2000. **60**(19): p. 5329-33.
99. Kulis, M. and M. Esteller, *DNA methylation and cancer.* Adv Genet. **70**: p. 27-56.
100. Nevadunsky, N.S., et al., *RUNX3 protein is overexpressed in human epithelial ovarian cancer.* Gynecol Oncol, 2009. **112**(2): p. 325-30.
101. Katsaros, D., et al., *Methylation of tumor suppressor gene p16 and prognosis of epithelial ovarian cancer.* Gynecol Oncol, 2004. **94**(3): p. 685-92.
102. Wong, T.S., et al., *The study of p16 and p15 gene methylation in head and neck squamous cell carcinoma and their quantitative evaluation in plasma by real-time PCR.* Eur J Cancer, 2003. **39**(13): p. 1881-7.
103. Furonaka, O., et al., *Aberrant methylation of p14(ARF), p15(INK4b) and p16(INK4a) genes and location of the primary site in pulmonary squamous cell carcinoma.* Pathol Int, 2004. **54**(8): p. 549-55.
104. Pfeifer, G.P., et al., *Methylation of the RASSF1A gene in human cancers.* Biol Chem, 2002. **383**(6): p. 907-14.
105. Widschwendter, M., et al., *DNA hypomethylation and ovarian cancer biology.* Cancer Res, 2004. **64**(13): p. 4472-80.
106. Rose, S.L., et al., *Epigenetic regulation of maspin expression in human ovarian carcinoma cells.* Gynecol Oncol, 2006. **102**(2): p. 319-24.
107. Czekierdowski, A., et al., *The role of CpG islands hypomethylation and abnormal expression of neuronal protein synuclein-gamma (SNCG) in ovarian cancer.* Neuro Endocrinol Lett, 2006. **27**(3): p. 381-6.

108. Balch, C., et al., *The epigenetics of ovarian cancer drug resistance and resensitization.* Am J Obstet Gynecol, 2004. **191**(5): p. 1552-72.
109. Teodoridis, J.M., et al., *CpG island methylation of DNA damage response genes in advanced ovarian cancer.* Cancer Res, 2005. **65**(19): p. 8961-7.
110. Wei, J., et al., *Epigenetic alterations of tumor marker microRNAs: towards new cancer therapies.* Drug News Perspect. **23**(10): p. 655-61.
111. Simpkins, S.B., et al., *MLH1 promoter methylation and gene silencing is the primary cause of microsatellite instability in sporadic endometrial cancers.* Hum Mol Genet, 1999. **8**(4): p. 661-6.
112. Deng, T. and Y. Zhang, *Possible involvement of activation of P53/P21 and demethylation of RUNX 3 in the cytotoxicity against Lovo cells induced by 5-Aza-2'-deoxycytidine.* Life Sci, 2009. **84**(9-10): p. 311-20.
113. Staub, J., et al., *Epigenetic silencing of HSulf-1 in ovarian cancer:implications in chemoresistance.* Oncogene, 2007. **26**(34): p. 4969-78.
114. Kunert, N. and A. Brehm, *Novel Mi-2 related ATP-dependent chromatin remodelers.* Epigenetics, 2009. **4**(4): p. 209-11.
115. Bouazoune, K., et al., *The dMi-2 chromodomains are DNA binding modules important for ATP-dependent nucleosome mobilization.* EMBO J, 2002. **21**(10): p. 2430-40.
116. Zhang, H., et al., *The CHD3 remodeler PICKLE promotes trimethylation of histone H3 lysine 27.* J Biol Chem, 2008. **283**(33): p. 22637-48.
117. Bouazoune, K. and A. Brehm, *ATP-dependent chromatin remodeling complexes in Drosophila.* Chromosome Res, 2006. **14**(4): p. 433-49.
118. Ogas, J., et al., *PICKLE is a CHD3 chromatin-remodeling factor that regulates the transition from embryonic to vegetative development in Arabidopsis.* Proc Natl Acad Sci U S A, 1999. **96**(24): p. 13839-44.
119. Gil, J., D. Bernard, and G. Peters, *Role of polycomb group proteins in stem cell self-renewal and cancer.* DNA Cell Biol, 2005. **24**(2): p. 117-25.
120. Bowen, N.J., et al., *Mi-2/NuRD: multiple complexes for many purposes.* Biochim Biophys Acta, 2004. **1677**(1-3): p. 52-7.
121. Marfella, C.G. and A.N. Imbalzano, *The Chd family of chromatin remodelers.* Mutat Res, 2007. **618**(1-2): p. 30-40.
122. Woodage, T., et al., *Characterization of the CHD family of proteins.* Proc Natl Acad Sci U S A, 1997. **94**(21): p. 11472-7.
123. Schmidt, D.R. and S.L. Schreiber, *Molecular association between ATR and two components of the nucleosome remodeling and deacetylating complex, HDAC2 and CHD4.* Biochemistry, 1999. **38**(44): p. 14711-7.
124. Ge, Q., et al., *Molecular analysis of a major antigenic region of the 240-kD protein of Mi-2 autoantigen.* J Clin Invest, 1995. **96**(4): p. 1730-7.
125. Airio, A., E. Pukkala, and H. Isomaki, *Elevated cancer incidence in patients with dermatomyositis: a population based study.* J Rheumatol, 1995. **22**(7): p. 1300-3.
126. Lemos, T. and E.A. Cavalheiro, *Suppression of pilocarpine-induced status epilepticus and the late development of epilepsy in rats.* Exp Brain Res, 1995. **102**(3): p. 423-8.

127. Goodarzi, A.A., T. Kurka, and P.A. Jeggo, *KAP-1 phosphorylation regulates CHD3 nucleosome remodeling during the DNA double-strand break response.* Nat Struct Mol Biol.
128. Stein, H., et al., *Identification of Hodgkin and Sternberg-reed cells as a unique cell type derived from a newly-detected small-cell population.* Int J Cancer, 1982. **30**(4): p. 445-59.
129. Bagchi, A., et al., *CHD5 is a tumor suppressor at human 1p36.* Cell, 2007. **128**(3): p. 459-75.
130. Sims, J.K. and P.A. Wade, *SnapShot: Chromatin remodeling: CHD.* Cell. **144**(4): p. 626-626 e1.
131. Heim, M.M., et al., *Differential modulation of chemosensitivity to alkylating agents and platinum compounds by DNA repair modulators in human lung cancer cell lines.* J Cancer Res Clin Oncol, 2000. **126**(4): p. 198-204.
132. Baker, V., *Oncogenes in gynecologic malignancy.* Curr Opin Obstet Gynecol, 1992. **4**(1): p. 75-80.
133. Bernardini, M.Q., et al., *Expression signatures of TP53 mutations in serous ovarian cancers.* BMC Cancer. **10**: p. 237.
134. McManus, D.T., et al., *p53 expression, mutation, and allelic deletion in ovarian cancer.* J Pathol, 1994. **174**(3): p. 159-68.
135. Yu, Y., et al., *NOEY2 (ARHI), an imprinted putative tumor suppressor gene in ovarian and breast carcinomas.* Proc Natl Acad Sci U S A, 1999. **96**(1): p. 214-9.
136. Schultz, D.C., et al., *Identification of two candidate tumor suppressor genes on chromosome 17p13.3.* Cancer Res, 1996. **56**(9): p. 1997-2002.
137. Ramakrishnan, R. and D.I. Gabrilovich, *Mechanism of synergistic effect of chemotherapy and immunotherapy of cancer.* Cancer Immunol Immunother. **60**(3): p. 419-23.
138. Baker, V.V., *Treatment options for ovarian cancer.* Clin Obstet Gynecol, 2001. **44**(3): p. 522-30.
139. Grignani, F., M. Martelli, and M. Tonato, *[Antimetabolites in the therapy of human acute leukemia. Mechanism of action and factors conditioning the cellular response to the drugs. 3. Cellular kinetics and chemotherapy].* Clin Ter, 1974. **69**(3): p. 263-79.
140. Coukos, G. and S.C. Rubin, *Chemotherapy resistance in ovarian cancer: new molecular perspectives.* Obstet Gynecol, 1998. **91**(5 Pt 1): p. 783-92.
141. van Jaarsveld, M.T., et al., *MicroRNAs in ovarian cancer biology and therapy resistance.* Int J Biochem Cell Biol. **42**(8): p. 1282-90.
142. Porro, A., et al., *Direct and coordinate regulation of ATP-binding cassette transporter genes by Myc factors generates specific transcription signatures that significantly affect the chemoresistance phenotype of cancer cells.* J Biol Chem. **285**(25): p. 19532-43.
143. Konopleva, M., et al., *The anti-apoptotic genes Bcl-X(L) and Bcl-2 are over-expressed and contribute to chemoresistance of non-proliferating leukaemic CD34+ cells.* Br J Haematol, 2002. **118**(2): p. 521-34.
144. Roberti, A., D. La Sala, and C. Cinti, *Multiple genetic and epigenetic interacting mechanisms contribute to clonally selection of drug-resistant tumors: current views and new therapeutic prospective.* J Cell Physiol, 2006. **207**(3): p. 571-81.

145. Bagadi, S.A., et al., *Clinical significance of promoter hypermethylation of RASSF1A, RARbeta2, BRCA1 and HOXA5 in breast cancers of Indian patients.* Life Sci, 2008. **82**(25-26): p. 1288-92.
146. Das, P.M. and R. Singal, *DNA methylation and cancer.* J Clin Oncol, 2004. **22**(22): p. 4632-42.
147. Aebi, S., et al., *Loss of DNA mismatch repair in acquired resistance to cisplatin.* Cancer Res, 1996. **56**(13): p. 3087-90.
148. Feng, Q., et al., *DNA hypermethylation, Her-2/neu overexpression and p53 mutations in ovarian carcinoma.* Gynecol Oncol, 2008. **111**(2): p. 320-9.
149. Girard, A. and G.J. Hannon, *Conserved themes in small-RNA-mediated transposon control.* Trends Cell Biol, 2008. **18**(3): p. 136-48.
150. Snove, O., Jr. and J.J. Rossi, *Toxicity in mice expressing short hairpin RNAs gives new insight into RNAi.* Genome Biol, 2006. **7**(8): p. 231.
151. Westbrook, T.F., F. Stegmeier, and S.J. Elledge, *Dissecting cancer pathways and vulnerabilities with RNAi.* Cold Spring Harb Symp Quant Biol, 2005. **70**: p. 435-44.
152. Siolas, D., et al., *Synthetic shRNAs as potent RNAi triggers.* Nat Biotechnol, 2005. **23**(2): p. 227-31.
153. Amarzguioui, M. and J.J. Rossi, *Principles of Dicer substrate (D-siRNA) design and function.* Methods Mol Biol, 2008. **442**: p. 3-10.
154. Hemann, M.T., et al., *An epi-allelic series of p53 hypomorphs created by stable RNAi produces distinct tumor phenotypes in vivo.* Nat Genet, 2003. **33**(3): p. 396-400.
155. Snove, O., Jr. and J.J. Rossi, *Expressing short hairpin RNAs in vivo.* Nat Methods, 2006. **3**(9): p. 689-95.
156. Whitehurst, A.W., et al., *Synthetic lethal screen identification of chemosensitizer loci in cancer cells.* Nature, 2007. **446**(7137): p. 815-9.
157. Singer, O. and I.M. Verma, *Applications of lentiviral vectors for shRNA delivery and transgenesis.* Curr Gene Ther, 2008. **8**(6): p. 483-8.
158. Stegmeier, F., et al., *A lentiviral microRNA-based system for single-copy polymerase II-regulated RNA interference in mammalian cells.* Proc Natl Acad Sci U S A, 2005. **102**(37): p. 13212-7.
159. Zender, L., et al., *An oncogenomics-based in vivo RNAi screen identifies tumor suppressors in liver cancer.* Cell, 2008. **135**(5): p. 852-64.
160. Ashrafi, K., et al., *Genome-wide RNAi analysis of Caenorhabditis elegans fat regulatory genes.* Nature, 2003. **421**(6920): p. 268-72.
161. Aza-Blanc, P., et al., *Identification of modulators of TRAIL-induced apoptosis via RNAi-based phenotypic screening.* Mol Cell, 2003. **12**(3): p. 627-37.
162. MacKeigan, J.P., L.O. Murphy, and J. Blenis, *Sensitized RNAi screen of human kinases and phosphatases identifies new regulators of apoptosis and chemoresistance.* Nat Cell Biol, 2005. **7**(6): p. 591-600.
163. Bric, A., et al., *Functional identification of tumor-suppressor genes through an in vivo RNA interference screen in a mouse lymphoma model.* Cancer Cell, 2009. **16**(4): p. 324-35.

164. Wu, K.J., et al., *[Comparison of transcription factors repressing epithelial phenotype in two different prostate cancer EMT models and its significance]*. Zhonghua Nan Ke Xue. **16**(2): p. 137-41.
165. Gregoire, L., et al., *Spontaneous malignant transformation of human ovarian surface epithelial cells in vitro.* Clin Cancer Res, 2001. **7**(12): p. 4280-7.
166. Tsao, S.W., et al., *Nonrandom chromosomal imbalances in human ovarian surface epithelial cells immortalized by HPV16-E6E7 viral oncogenes.* Cancer Genet Cytogenet, 2001. **130**(2): p. 141-9.
167. Cooper, M.T., A.W. Conant, and J.A. Kennison, *Molecular genetic analysis of Chd3 and polytene chromosome region 76B-D in Drosophila melanogaster.* Genetics. **185**(3): p. 811-22.
168. De Toni, F., et al., *A crosstalk between the Wnt and the adhesion-dependent signaling pathways governs the chemosensitivity of acute myeloid leukemia.* Oncogene, 2006. **25**(22): p. 3113-22.
169. Feng, Z., et al., *Mammalian target of rapamycin regulates expression of beta-catenin in hepatocellular carcinoma.* Hum Pathol. **42**(5): p. 659-68.
170. Flahaut, M., et al., *The Wnt receptor FZD1 mediates chemoresistance in neuroblastoma through activation of the Wnt/beta-catenin pathway.* Oncogene, 2009. **28**(23): p. 2245-56.
171. Li, L.N., et al., *Artesunate attenuates the growth of human colorectal carcinoma and inhibits hyperactive Wnt/beta-catenin pathway.* Int J Cancer, 2007. **121**(6): p. 1360-5.
172. Su, H.Y., et al., *Epigenetic silencing of SFRP5 is related to malignant phenotype and chemoresistance of ovarian cancer through Wnt signaling pathway.* Int J Cancer. **127**(3): p. 555-67.
173. Gatcliffe, T.A., et al., *Wnt signaling in ovarian tumorigenesis.* Int J Gynecol Cancer, 2008. **18**(5): p. 954-62.
174. Konstantinopoulos, P.A., et al., *Integrated analysis of multiple microarray datasets identifies a reproducible survival predictor in ovarian cancer.* PLoS ONE. **6**(3): p. e18202.
175. Huang da, W., B.T. Sherman, and R.A. Lempicki, *Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources.* Nat Protoc, 2009. **4**(1): p. 44-57.
176. Huang da, W., B.T. Sherman, and R.A. Lempicki, *Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists.* Nucleic Acids Res, 2009. **37**(1): p. 1-13.
177. Hough, C.D., et al., *Large-scale serial analysis of gene expression reveals genes differentially expressed in ovarian cancer.* Cancer Res, 2000. **60**(22): p. 6281-7.
178. Feinberg, A.P., *The epigenetics of cancer etiology.* Semin Cancer Biol, 2004. **14**(6): p. 427-32.
179. Fojo, T. and S. Bates, *Strategies for reversing drug resistance.* Oncogene, 2003. **22**(47): p. 7512-23.
180. Montemurro, F., et al., *High-dose chemotherapy with hematopoietic stem-cell transplantation for breast cancer: current status, future trends.* Clin Breast Cancer, 2000. **1**(3): p. 197-209; discussion 210.

181. Itamochi, H., et al., *Low proliferation activity may be associated with chemoresistance in clear cell carcinoma of the ovary.* Obstet Gynecol, 2002. **100**(2): p. 281-7.
182. Kunert, N., et al., *dMec: a novel Mi-2 chromatin remodelling complex involved in transcriptional repression.* EMBO J, 2009. **28**(5): p. 533-44.
183. Song, B., et al., *Molecular mechanism of chemoresistance by miR-215 in osteosarcoma and colon cancer cells.* Mol Cancer. **9**: p. 96.
184. Nam, E.J. and Y.T. Kim, *Alteration of cell-cycle regulation in epithelial ovarian cancer.* Int J Gynecol Cancer, 2008. **18**(6): p. 1169-82.
185. Delavaine, L. and N.B. La Thangue, *Control of E2F activity by p21Waf1/Cip1.* Oncogene, 1999. **18**(39): p. 5381-92.
186. Vijayalaxmi and G. Obe, *Controversial cytogenetic observations in mammalian somatic cells exposed to radiofrequency radiation.* Radiat Res, 2004. **162**(5): p. 481-96.
187. Ljungkvist, A.S., et al., *Vascular architecture, hypoxia, and proliferation in first-generation xenografts of human head-and-neck squamous cell carcinomas.* Int J Radiat Oncol Biol Phys, 2002. **54**(1): p. 215-28.
188. Tannock, I.F., *Responsibility in advertising: an oncologist's view.* N Engl J Med, 1979. **301**(18): p. 1004.
189. Srinivasan, L., X. Pan, and M.L. Atchison, *Transient requirements of YY1 expression for PcG transcriptional repression and phenotypic rescue.* J Cell Biochem, 2005. **96**(4): p. 689-99.
190. Abel, E.V. and A.E. Aplin, *FOXD3 is a mutant B-RAF-regulated inhibitor of G(1)-S progression in melanoma cells.* Cancer Res. **70**(7): p. 2891-900.
191. Katschinski, D.M., et al., *Role of tumor necrosis factor alpha in hyperthermia-induced apoptosis of human leukemia cells.* Cancer Res, 1999. **59**(14): p. 3404-10.
192. Kamalakaran, S., et al., *Methylation detection oligonucleotide microarray analysis: a high-resolution method for detection of CpG island methylation.* Nucleic Acids Res, 2009. **37**(12): p. e89.
193. Licht, T., M.M. Gottesman, and I. Pastan, *Transfer of the MDR1 (multidrug resistance) gene: protection of hematopoietic cells from cytotoxic chemotherapy, and selection of transduced cells in vivo.* Cytokines Mol Ther, 1995. **1**(1): p. 11-20.
194. Cole, S.P., et al., *Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells.* Cancer Res, 1994. **54**(22): p. 5902-10.
195. Paumi, C.M., et al., *Role of multidrug resistance protein 1 (MRP1) and glutathione S-transferase A1-1 in alkylating agent resistance. Kinetics of glutathione conjugate formation and efflux govern differential cellular sensitivity to chlorambucil versus melphalan toxicity.* J Biol Chem, 2001. **276**(11): p. 7952-6.
196. Kelland, L.R., et al., *Mechanism-related circumvention of acquired cis-diamminedichloroplatinum(II) resistance using two pairs of human ovarian carcinoma cell lines by ammine/amine platinum(IV) dicarboxylates.* Cancer Res, 1992. **52**(14): p. 3857-64.

197. Rischin, D. and V. Ling, *Ormaplatin resistance is associated with decreased accumulation of its platinum (II) analogue, dichloro(D,L-trans)1,2-diaminocyclohexaneplatinum (II).* Br J Cancer, 1996. **74**(4): p. 590-6.
198. Rixe, O., et al., *Oxaliplatin, tetraplatin, cisplatin, and carboplatin: spectrum of activity in drug-resistant cell lines and in the cell lines of the National Cancer Institute's Anticancer Drug Screen panel.* Biochem Pharmacol, 1996. **52**(12): p. 1855-65.
199. Itoh, K., et al., *Successful treatment with nedaplatin in patients with ovarian cancer that recurred after platinum-containing chemotherapy: report of two cases.* Jpn J Clin Oncol, 1998. **28**(5): p. 343-6.
200. Ichikawa, Y., et al., *Inactivation of p16/CDKN2 and p15/MTS2 genes in different histological types and clinical stages of primary ovarian tumors.* Int J Cancer, 1996. **69**(6): p. 466-70.
201. Shen, D., I. Pastan, and M.M. Gottesman, *Cross-resistance to methotrexate and metals in human cisplatin-resistant cell lines results from a pleiotropic defect in accumulation of these compounds associated with reduced plasma membrane binding proteins.* Cancer Res, 1998. **58**(2): p. 268-75.
202. Pennington, K., et al., *Too much of a good thing: suicide prevention promotes chemoresistance in ovarian carcinoma.* Curr Cancer Drug Targets. **10**(6): p. 575-83.
203. Gosland, M., et al., *Insights into mechanisms of cisplatin resistance and potential for its clinical reversal.* Pharmacotherapy, 1996. **16**(1): p. 16-39.
204. Itamochi, H., *Targeted therapies in epithelial ovarian cancer: Molecular mechanisms of action.* World J Biol Chem. **1**(7): p. 209-20.
205. Sharom, F.J., *ABC multidrug transporters: structure, function and role in chemoresistance.* Pharmacogenomics, 2008. **9**(1): p. 105-27.
206. Dicato, M., et al., *Multidrug resistance: molecular and clinical aspects.* Cytokines Cell Mol Ther, 1997. **3**(2): p. 91-9.
207. Vaupel, P., A. Mayer, and M. Hockel, *Tumor hypoxia and malignant progression.* Methods Enzymol, 2004. **381**: p. 335-54.
208. Carey, L.A., et al., *The triple negative paradox: primary tumor chemosensitivity of breast cancer subtypes.* Clin Cancer Res, 2007. **13**(8): p. 2329-34.
209. Blumenthal, R.D. and D.M. Goldenberg, *Methods and goals for the use of in vitro and in vivo chemosensitivity testing.* Mol Biotechnol, 2007. **35**(2): p. 185-97.
210. Cheng, J.Q., et al., *Role of X-linked inhibitor of apoptosis protein in chemoresistance in ovarian cancer: possible involvement of the phosphoinositide-3 kinase/Akt pathway.* Drug Resist Updat, 2002. **5**(3-4): p. 131-46.
211. Yuan, Z.Q., et al., *Frequent activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer.* Oncogene, 2000. **19**(19): p. 2324-30.
212. Sardi, I., et al., *Molecular profiling of high-risk neuroblastoma by cDNA array.* Int J Mol Med, 2002. **9**(5): p. 541-5.
213. Schneiderman, D., et al., *Sustained suppression of Fas ligand expression in cisplatin-resistant human ovarian surface epithelial cancer cells.* Apoptosis, 1999. **4**(4): p. 271-81.

214. Fearnhead, H.O., et al., *Oncogene-dependent apoptosis is mediated by caspase-9.* Proc Natl Acad Sci U S A, 1998. **95**(23): p. 13664-9.
215. Kurrey, N.K., et al., *Snail and slug mediate radioresistance and chemoresistance by antagonizing p53-mediated apoptosis and acquiring a stem-like phenotype in ovarian cancer cells.* Stem Cells, 2009. **27**(9): p. 2059-68.
216. Hajra, K.M., L. Tan, and J.R. Liu, *Defective apoptosis underlies chemoresistance in ovarian cancer.* Adv Exp Med Biol, 2008. **622**: p. 197-208.
217. Campioni, M., et al., *Role of Apaf-1, a key regulator of apoptosis, in melanoma progression and chemoresistance.* Exp Dermatol, 2005. **14**(11): p. 811-8.
218. Bai, J., et al., *Predominant Bcl-XL knockdown disables antiapoptotic mechanisms: tumor necrosis factor-related apoptosis-inducing ligand-based triple chemotherapy overcomes chemoresistance in pancreatic cancer cells in vitro.* Cancer Res, 2005. **65**(6): p. 2344-52.
219. Rogakou, E.P., et al., *Initiation of DNA fragmentation during apoptosis induces phosphorylation of H2AX histone at serine 139.* J Biol Chem, 2000. **275**(13): p. 9390-5.
220. Rogakou, E.P., et al., *Rapid histone extraction for electrophoretic analysis.* Biotechniques, 2000. **28**(1): p. 38-40, 42, 46.
221. Rogakou, E.P., et al., *DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139.* J Biol Chem, 1998. **273**(10): p. 5858-68.
222. Thornberry, N.A. and Y. Lazebnik, *Caspases: enemies within.* Science, 1998. **281**(5381): p. 1312-6.
223. Matzinger, P., *The JAM test. A simple assay for DNA fragmentation and cell death.* J Immunol Methods, 1991. **145**(1-2): p. 185-92.
224. Falcieri, E., et al., *The protein kinase inhibitor staurosporine induces morphological changes typical of apoptosis in MOLT-4 cells without concomitant DNA fragmentation.* Biochem Biophys Res Commun, 1993. **193**(1): p. 19-25.
225. Lazebnik, Y.A., et al., *Nuclear events of apoptosis in vitro in cell-free mitotic extracts: a model system for analysis of the active phase of apoptosis.* J Cell Biol, 1993. **123**(1): p. 7-22.
226. Campisi, J., *Aging, tumor suppression and cancer: high wire-act!* Mech Ageing Dev, 2005. **126**(1): p. 51-8.
227. Dimri, G.P., *What has senescence got to do with cancer?* Cancer Cell, 2005. **7**(6): p. 505-12.
228. Johnstone, R.W., A.A. Ruefli, and S.W. Lowe, *Apoptosis: a link between cancer genetics and chemotherapy.* Cell, 2002. **108**(2): p. 153-64.
229. Lowe, S.W., et al., *p53-dependent apoptosis modulates the cytotoxicity of anticancer agents.* Cell, 1993. **74**(6): p. 957-67.
230. Brown, J.M. and G. Wilson, *Apoptosis genes and resistance to cancer therapy: what does the experimental and clinical data tell us?* Cancer Biol Ther, 2003. **2**(5): p. 477-90.
231. Brown, J.M. and L.D. Attardi, *The role of apoptosis in cancer development and treatment response.* Nat Rev Cancer, 2005. **5**(3): p. 231-7.
232. Brown, G.D., *Sensing necrosis with Mincle.* Nat Immunol, 2008. **9**(10): p. 1099-100.

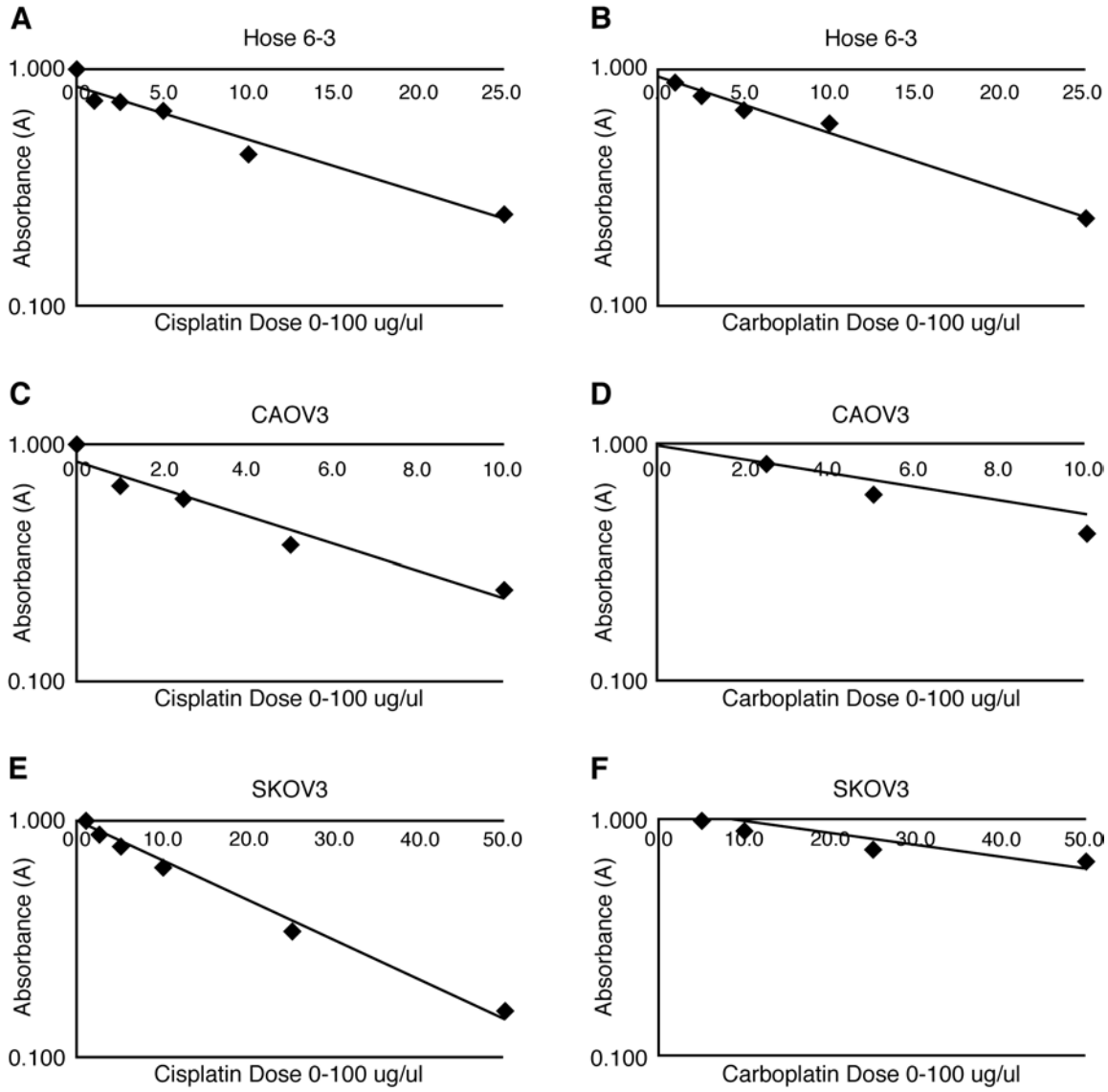
233. Lauber, K., et al., *Clearance of apoptotic cells: getting rid of the corpses.* Mol Cell, 2004. **14**(3): p. 277-87.
234. Stiewe, T. and B.M. Putzer, *Role of p73 in malignancy: tumor suppressor or oncogene?* Cell Death Differ, 2002. **9**(3): p. 237-45.
235. Stiewe, T., C.C. Theseling, and B.M. Putzer, *Transactivation-deficient Delta TA-p73 inhibits p53 by direct competition for DNA binding: implications for tumorigenesis.* J Biol Chem, 2002. **277**(16): p. 14177-85.
236. Urist, M., et al., *p73 induction after DNA damage is regulated by checkpoint kinases Chk1 and Chk2.* Genes Dev, 2004. **18**(24): p. 3041-54.
237. Katz, E., et al., *An in vitro model that recapitulates the epithelial to mesenchymal transition (EMT) in human breast cancer.* PLoS ONE. **6**(2): p. e17083.
238. Polyak, K., et al., *Breast tumor heterogeneity: causes and consequences.* Breast Cancer Res, 2009. **11 Suppl 1**: p. S18.
239. Thiery, J.P., *Epithelial-mesenchymal transitions in tumour progression.* Nat Rev Cancer, 2002. **2**(6): p. 442-54.
240. Ngan, C.Y., et al., *A multivariate analysis of adhesion molecules expression in assessment of colorectal cancer.* J Surg Oncol, 2007. **95**(8): p. 652-62.
241. Dorudi, S. and I.R. Hart, *Mechanisms underlying invasion and metastasis.* Curr Opin Oncol, 1993. **5**(1): p. 130-5.
242. Peinado, H., D. Olmeda, and A. Cano, *Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype?* Nat Rev Cancer, 2007. **7**(6): p. 415-28.
243. Singh, A. and J. Settleman, *EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer.* Oncogene. **29**(34): p. 4741-51.
244. Gomes, L.R., et al., *Epithelial-Mesenchymal Transition: Implications in Cancer Progression and Metastasis.* Curr Pharm Biotechnol.
245. Kwok, W.K., et al., *Up-regulation of TWIST in prostate cancer and its implication as a therapeutic target.* Cancer Res, 2005. **65**(12): p. 5153-62.
246. Yang, A.D., et al., *Chronic oxaliplatin resistance induces epithelial-to-mesenchymal transition in colorectal cancer cell lines.* Clin Cancer Res, 2006. **12**(14 Pt 1): p. 4147-53.
247. Shah, A.N., et al., *Development and characterization of gemcitabine-resistant pancreatic tumor cells.* Ann Surg Oncol, 2007. **14**(12): p. 3629-37.
248. Hosono, S., et al., *Expression of Twist increases the risk for recurrence and for poor survival in epithelial ovarian carcinoma patients.* Br J Cancer, 2007. **96**(2): p. 314-20.
249. Thomson, S., et al., *Epithelial to mesenchymal transition is a determinant of sensitivity of non-small-cell lung carcinoma cell lines and xenografts to epidermal growth factor receptor inhibition.* Cancer Res, 2005. **65**(20): p. 9455-62.
250. Frederick, B.A., et al., *Epithelial to mesenchymal transition predicts gefitinib resistance in cell lines of head and neck squamous cell carcinoma and non-small cell lung carcinoma.* Mol Cancer Ther, 2007. **6**(6): p. 1683-91.
251. Fuchs, B.C., et al., *Epithelial-to-mesenchymal transition and integrin-linked kinase mediate sensitivity to epidermal growth factor receptor inhibition in human hepatoma cells.* Cancer Res, 2008. **68**(7): p. 2391-9.

252. Cifone, M.A. and I.J. Fidler, *Correlation of patterns of anchorage-independent growth with in vivo behavior of cells from a murine fibrosarcoma.* Proc Natl Acad Sci U S A, 1980. **77**(2): p. 1039-43.
253. Montagnier, L. and I. Macpherson, *[SELECTIVE DEVELOPMENT IN AGAR OF HAMSTER CELLS TRANSFORMED BY THE POLYOMA VIRUS].* C R Hebd Seances Acad Sci, 1964. **258**: p. 4171-3.
254. Ridley, A.J., et al., *Cell migration: integrating signals from front to back.* Science, 2003. **302**(5651): p. 1704-9.
255. Bonnomet, A., et al., *Epithelial-to-mesenchymal transitions and circulating tumor cells.* J Mammary Gland Biol Neoplasia. **15**(2): p. 261-73.
256. Liang, C.C., A.Y. Park, and J.L. Guan, *In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro.* Nat Protoc, 2007. **2**(2): p. 329-33.
257. Knutson, J.R., et al., *CD44/chondroitin sulfate proteoglycan and alpha 2 beta 1 integrin mediate human melanoma cell migration on type IV collagen and invasion of basement membranes.* Mol Biol Cell, 1996. **7**(3): p. 383-96.
258. Gavert, N. and A. Ben-Ze'ev, *Coordinating changes in cell adhesion and phenotype during EMT-like processes in cancer.* F1000 Biol Rep. **2**: p. 86.
259. Sarkar, F.H., et al., *Pancreatic cancer stem cells and EMT in drug resistance and metastasis.* Minerva Chir, 2009. **64**(5): p. 489-500.
260. Wang, Z., et al., *Targeting miRNAs involved in cancer stem cell and EMT regulation: An emerging concept in overcoming drug resistance.* Drug Resist Updat. **13**(4-5): p. 109-18.
261. Hiscox, S., et al., *Tamoxifen resistance in MCF7 cells promotes EMT-like behaviour and involves modulation of beta-catenin phosphorylation.* Int J Cancer, 2006. **118**(2): p. 290-301.
262. Werbowetski-Ogilvie, T.E., et al., *Characterization of human embryonic stem cells with features of neoplastic progression.* Nat Biotechnol, 2009. **27**(1): p. 91-7.
263. Kahn, P. and S.I. Shin, *Cellular tumorigenicity in nude mice. Test of associations among loss of cell-surface fibronectin, anchorage independence, and tumor-forming ability.* J Cell Biol, 1979. **82**(1): p. 1-16.
264. Le Devedec, S.E., et al., *An improved model to study tumor cell autonomous metastasis programs using MTLn3 cells and the Rag2(-/-) gammac (-/-) mouse.* Clin Exp Metastasis, 2009. **26**(7): p. 673-84.
265. Thiery, J.P., et al., *Epithelial-mesenchymal transitions in development and disease.* Cell, 2009. **139**(5): p. 871-90.
266. Chen, X., et al., *Epithelial Mesenchymal Transition and Hedgehog Signaling Activation are Associated with Chemoresistance and Invasion of Hepatoma Subpopulations.* J Hepatol.
267. Terauchi, M., et al., *Possible involvement of TWIST in enhanced peritoneal metastasis of epithelial ovarian carcinoma.* Clin Exp Metastasis, 2007. **24**(5): p. 329-39.
268. Chang, T.H., et al., *Slug confers resistance to the epidermal growth factor receptor tyrosine kinase inhibitor.* Am J Respir Crit Care Med. **183**(8): p. 1071-9.

269. Rho, J.K., et al., *Epithelial to mesenchymal transition derived from repeated exposure to gefitinib determines the sensitivity to EGFR inhibitors in A549, a non-small cell lung cancer cell line.* Lung Cancer, 2009. **63**(2): p. 219-26.
270. Iwatsuki, H. and M. Suda, *Seven kinds of intermediate filament networks in the cytoplasm of polarized cells: structure and function.* Acta Histochem Cytochem. **43**(2): p. 19-31.
271. Wang, X., et al., *Identification of a novel function of TWIST, a bHLH protein, in the development of acquired taxol resistance in human cancer cells.* Oncogene, 2004. **23**(2): p. 474-82.
272. Safina, A., E. Vandette, and A.V. Bakin, *ALK5 promotes tumor angiogenesis by upregulating matrix metalloproteinase-9 in tumor cells.* Oncogene, 2007. **26**(17): p. 2407-22.
273. Skirnisdottir, I., K. Lindborg, and B. Sorbe, *Adjuvant chemotherapy with carboplatin and taxane compared with single drug carboplatin in early stage epithelial ovarian carcinoma.* Oncol Rep, 2007. **18**(5): p. 1249-56.
274. Fendrich, V., et al., *Unique expression pattern of the EMT markers Snail, Twist and E-cadherin in benign and malignant parathyroid neoplasia.* Eur J Endocrinol, 2009. **160**(4): p. 695-703.
275. Kolfschoten, G.M., et al., *Drug resistance features and S-phase fraction as possible determinants for drug response in a panel of human ovarian cancer xenografts.* Br J Cancer, 2000. **83**(7): p. 921-7.
276. Mann, S.C., P.A. Andrews, and S.B. Howell, *Comparison of lipid content, surface membrane fluidity, and temperature dependence of cis-diamminedichloroplatinum(II) accumulation in sensitive and resistant human ovarian carcinoma cells.* Anticancer Res, 1988. **8**(6): p. 1211-5.
277. Azouz, A., et al., *Immunoinflammatory responses and fibrogenesis.* Med Electron Microsc, 2004. **37**(3): p. 141-8.
278. Christen, R.D., et al., *Signaling and drug sensitivity.* Cancer Metastasis Rev, 1994. **13**(2): p. 175-89.
279. Fujii, R., et al., *Active efflux system for cisplatin in cisplatin-resistant human KB cells.* Jpn J Cancer Res, 1994. **85**(4): p. 426-33.
280. Bianco, R., et al., *Vascular endothelial growth factor receptor-1 contributes to resistance to anti-epidermal growth factor receptor drugs in human cancer cells.* Clin Cancer Res, 2008. **14**(16): p. 5069-80.
281. Normanno, N., et al., *The role of EGF-related peptides in tumor growth.* Front Biosci, 2001. **6**: p. D685-707.
282. Loh, S.Y., et al., *Reduced drug accumulation as a major mechanism of acquired resistance to cisplatin in a human ovarian carcinoma cell line: circumvention studies using novel platinum (II) and (IV) ammine/amine complexes.* Br J Cancer, 1992. **66**(6): p. 1109-15.
283. Gadducci, A., et al., *Molecular mechanisms of apoptosis and chemosensitivity to platinum and paclitaxel in ovarian cancer: biological data and clinical implications.* Eur J Gynaecol Oncol, 2002. **23**(5): p. 390-6.
284. Groth, A., et al., *Regulation of replication fork progression through histone supply and demand.* Science, 2007. **318**(5858): p. 1928-31.

285. Verhagen, A.M., et al., *HtrA2 promotes cell death through its serine protease activity and its ability to antagonize inhibitor of apoptosis proteins.* J Biol Chem, 2002. **277**(1): p. 445-54.
286. Dong, J., et al., *Bcl-2 upregulation induced by miR-21 via a direct interaction is associated with apoptosis and chemoresistance in MIA PaCa-2 pancreatic cancer cells.* Arch Med Res. **42**(1): p. 8-14.
287. Choi, K.C., et al., *Estradiol up-regulates antiapoptotic Bcl-2 messenger ribonucleic acid and protein in tumorigenic ovarian surface epithelium cells.* Endocrinology, 2001. **142**(6): p. 2351-60.
288. Berge, E.O., et al., *Identification and characterization of retinoblastoma gene mutations disturbing apoptosis in human breast cancers.* Mol Cancer. **9**: p. 173.
289. Dimanche-Boitrel, M.T., et al., *Contribution of the cyclin-dependent kinase inhibitor p27KIP1 to the confluence-dependent resistance of HT29 human colon carcinoma cells.* Int J Cancer, 1998. **77**(5): p. 796-802.
290. Miller, A.M. and K.A. Nasmyth, *Role of DNA replication in the repression of silent mating type loci in yeast.* Nature, 1984. **312**(5991): p. 247-51.
291. Thiery, J.P. and D. Chopin, *Epithelial cell plasticity in development and tumor progression.* Cancer Metastasis Rev, 1999. **18**(1): p. 31-42.
292. Yin, G., et al., *TWISTing stemness, inflammation and proliferation of epithelial ovarian cancer cells through MIR199A2/214.* Oncogene. **29**(24): p. 3545-53.
293. Helleman, J., et al., *Pathway analysis of gene lists associated with platinum-based chemotherapy resistance in ovarian cancer: the big picture.* Gynecol Oncol. **117**(2): p. 170-6.
294. Wang, Z., et al., *Acquisition of epithelial-mesenchymal transition phenotype of gemcitabine-resistant pancreatic cancer cells is linked with activation of the notch signaling pathway.* Cancer Res, 2009. **69**(6): p. 2400-7.
295. Lei, W.W., et al., *Histone deacetylase 1 and 2 differentially regulate apoptosis by opposing effects on extracellular signal-regulated kinase 1/2.* Cell Death Dis. **1**(5): p. e44.
296. Pakala, S.B., et al., *TGF-beta1 signaling targets metastasis-associated protein 1, a new effector in epithelial cells.* Oncogene. **30**(19): p. 2230-41.
297. Lei, W., et al., *Histone deacetylase 1 is required for transforming growth factor-beta1-induced epithelial-mesenchymal transition.* Int J Biochem Cell Biol. **42**(9): p. 1489-97.
298. Noh, H., et al., *Histone deacetylase-2 is a key regulator of diabetes- and transforming growth factor-beta1-induced renal injury.* Am J Physiol Renal Physiol, 2009. **297**(3): p. F729-39.
299. Srivastava, R.K., R. Kurzrock, and S. Shankar, *MS-275 sensitizes TRAIL-resistant breast cancer cells, inhibits angiogenesis and metastasis, and reverses epithelial-mesenchymal transition in vivo.* Mol Cancer Ther. **9**(12): p. 3254-66.
300. Yoshikawa, M., et al., *Inhibition of histone deacetylase activity suppresses epithelial-to-mesenchymal transition induced by TGF-beta1 in human renal epithelial cells.* J Am Soc Nephrol, 2007. **18**(1): p. 58-65.
301. Xue, Y., et al., *NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities.* Mol Cell, 1998. **2**(6): p. 851-61.

Appendix 1 In vitro cell line carboplatin and cisplatin survival curves



Appendix 1: Panel A-F Growth sensitivity of the ovarian cancer cell lines (Hose 6-3, CAOV3, and SKOV3) to platinum drugs. These figures illustrate the three ovarian cancer cell lines used for functional validation. These cell lines were characterized for carboplatin sensitivity from 0-100uM concentrations to determine IC20, IC50 and IC80 values. These cell lines represent a range of sensitivity to carboplatin. The x-axis

represents the dose range for carboplatin and the y-axis lists the survival in log scale. Hose 6-3 (**A, B**), CAOV3 (**C, D**), SKOV3 (**E, F**).

Appendix 2-1 pLKO and pGIPZ shRNA vector information

pLKO *CHD3* Vector Information

Accessions	NM_001005271
Vector Name	pLKO.1
Vector Type	Lentiviral
Antibiotic Information	Carbenicillin (Concentration: 100 µg/ml, Resistance Range: 100-100 µg/ml)

Hairpin sequence:
CCGGCCTCCCACACTGCCAAGTATACTCGAGTATACTTGGCAGTGTGGGAGGTTTTTG

Mature Sense: CCTCCCACACTGCCAAGTATA
Mature Antisense: TATACTTGGCAGTGTGGGAGG

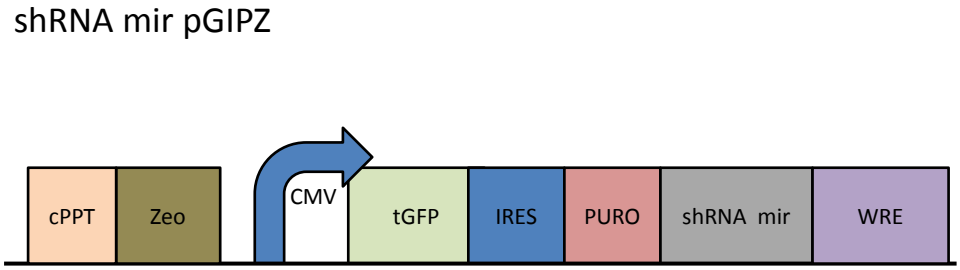
pGIPZ *CHD3* Vector Information

Accessions	NM_001005271
Vector Name	pGIPZ
Vector Type	Lentiviral
Antibiotic Information	Zeocin (Concentration: 25 µg/ml, Resistant Range: 25-25 µg/ml) Ampicillin (Concentration: 100 µg/ml, Resistant Range: 100-100 µg/ml)

Hairpin sequence:
TGCTGTTGACAGTGAGCGCCTGGCACAGAAGACATGACTATAGTGAAGCCACAGATGTATAGTCATGTCTTCTGTGCCAGATGCCTACTGCCTCGGA
Mature Sense: TGGCACAGAAGACATGACT
Mature Antisense: AGTCATGTCTTCTGTGCCA

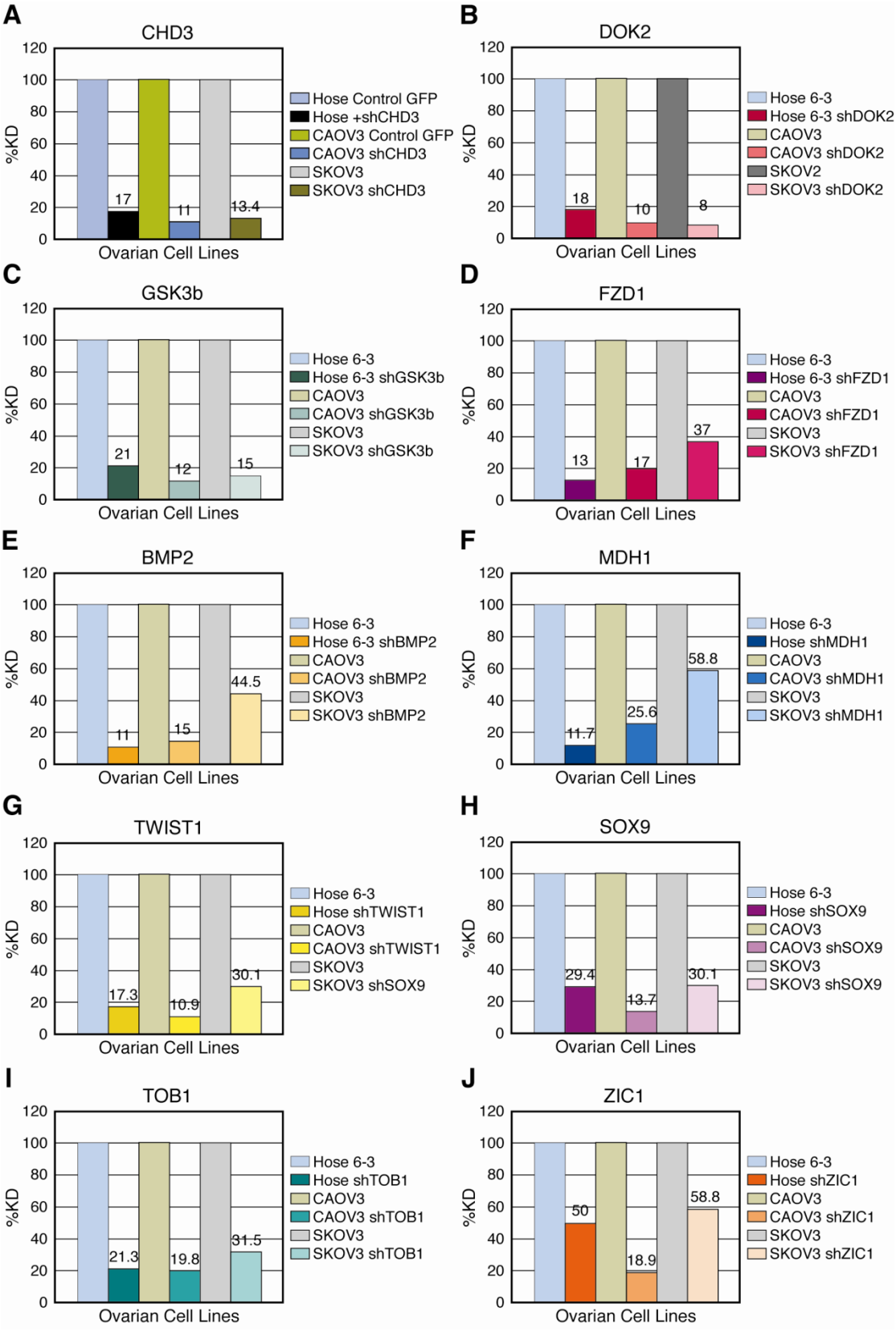
Figure 2-1 This figure represents the two different shRNA Lentiviral based vectors that we used for further analysis of *CHD3* clone. Lentiviral-mediated gene transfer was performed following the standard infection/transfection protocol (Open Biosystems).

Appendix 2-2 pGIPZ Vector Features



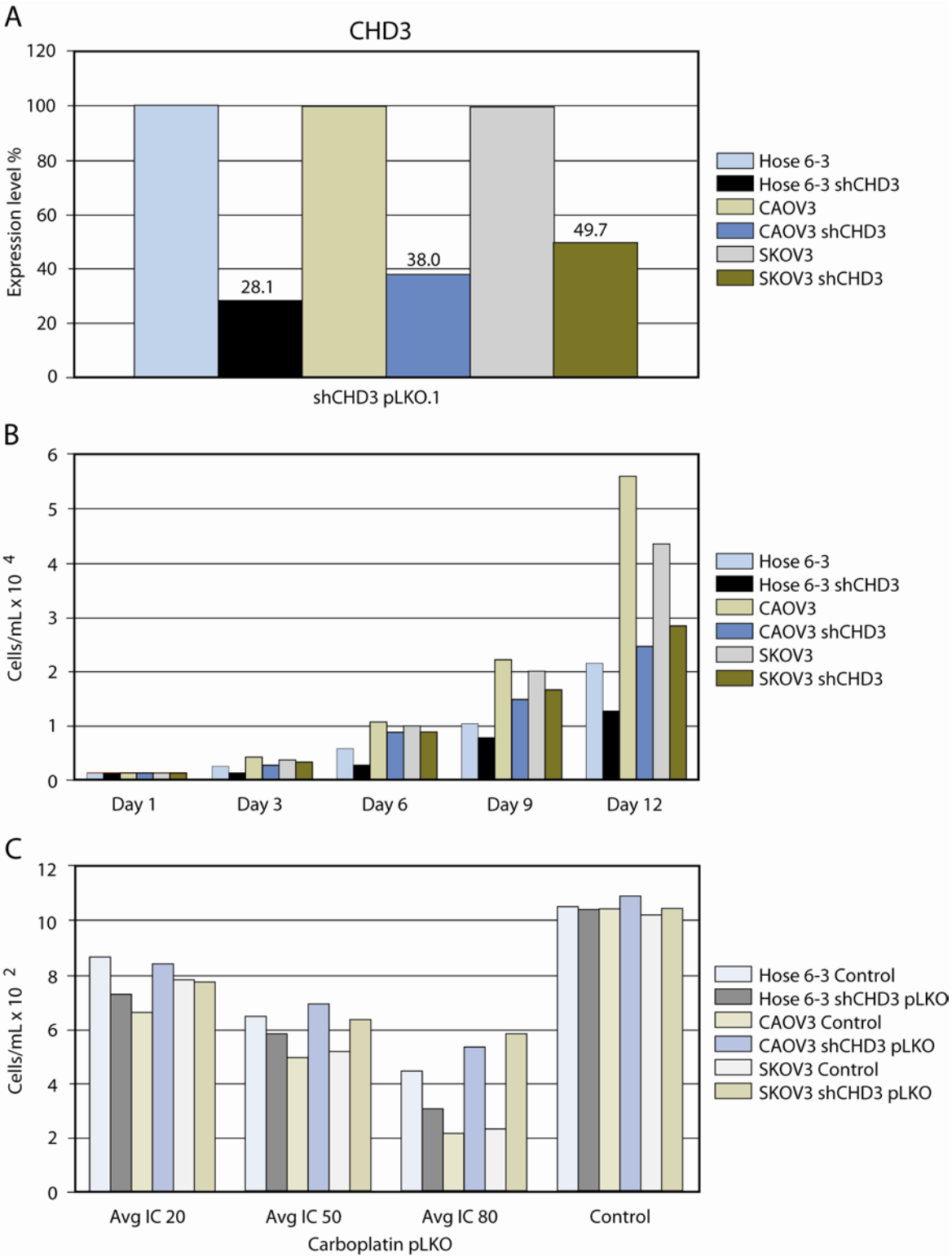
Appendix 2-2: This figure is a representation of the pGIPZ vector and shRNA construct.
cPPT: Central polypurine tract, helps translocate into nucleus of nondividing cells
Zeo: bacterial selectable marker
CMV: RNA Polymerase II promoter
GFP: Marker to track shRNAmir expression
IRES: internal ribosome entry site, translation of mRNA
Puro: Mammalian selectable marker
WRE: Enhances the stability and translation of transcripts

Appendix 2-3 Single Clone Validation RT-Q-PCR



Appendix 2-3: shows Lentiviral encoded shRNAs MiR-30 microRNAs from Open BioSystems independently validated for efficient transcript knockdown using RT-Q-PCR. **Panel A-J** are bar plots of RT-Q-PCR analysis of the shRNA infections calculating the efficiency of shRNA induced knockdown relative to the control non target shRNA infection. The Y axis is the percentage transcript with the non target shown as 100% by definition of being the reference. Each bar represents one of the infections in one of the three cell lines used. Inset on top of the bars for cell lines is the level of transcript determined to be remaining after shRNA induced knockdown.

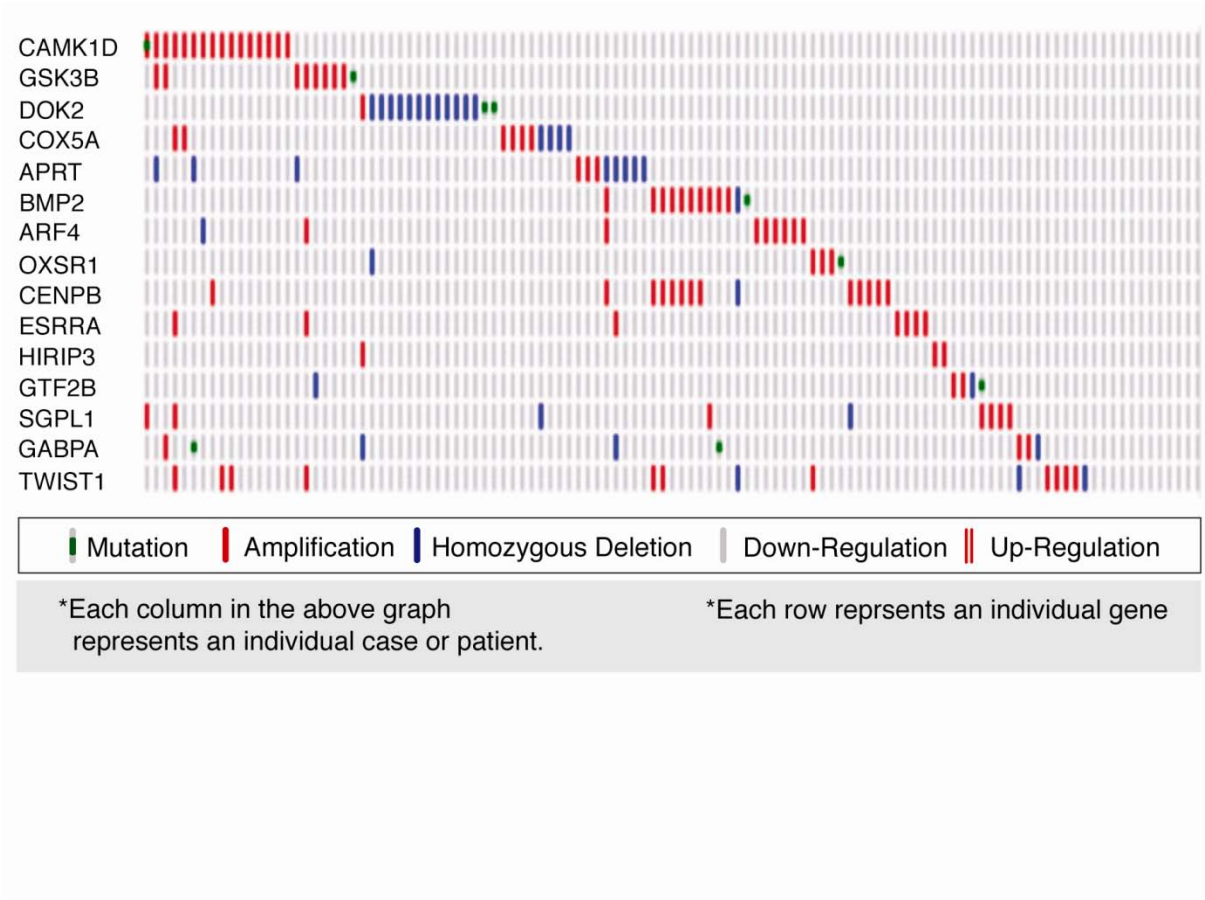
Appendix 2-4 pLKO RT-Q-PCR shRNA *CHD3* Validation and Growth



Appendix 2-4: shows validation of specificity of the pLKO shRNA clones targeting *CHD3*. This graph represents a bar plot of RT-Q-PCR analysis of the shRNA infections

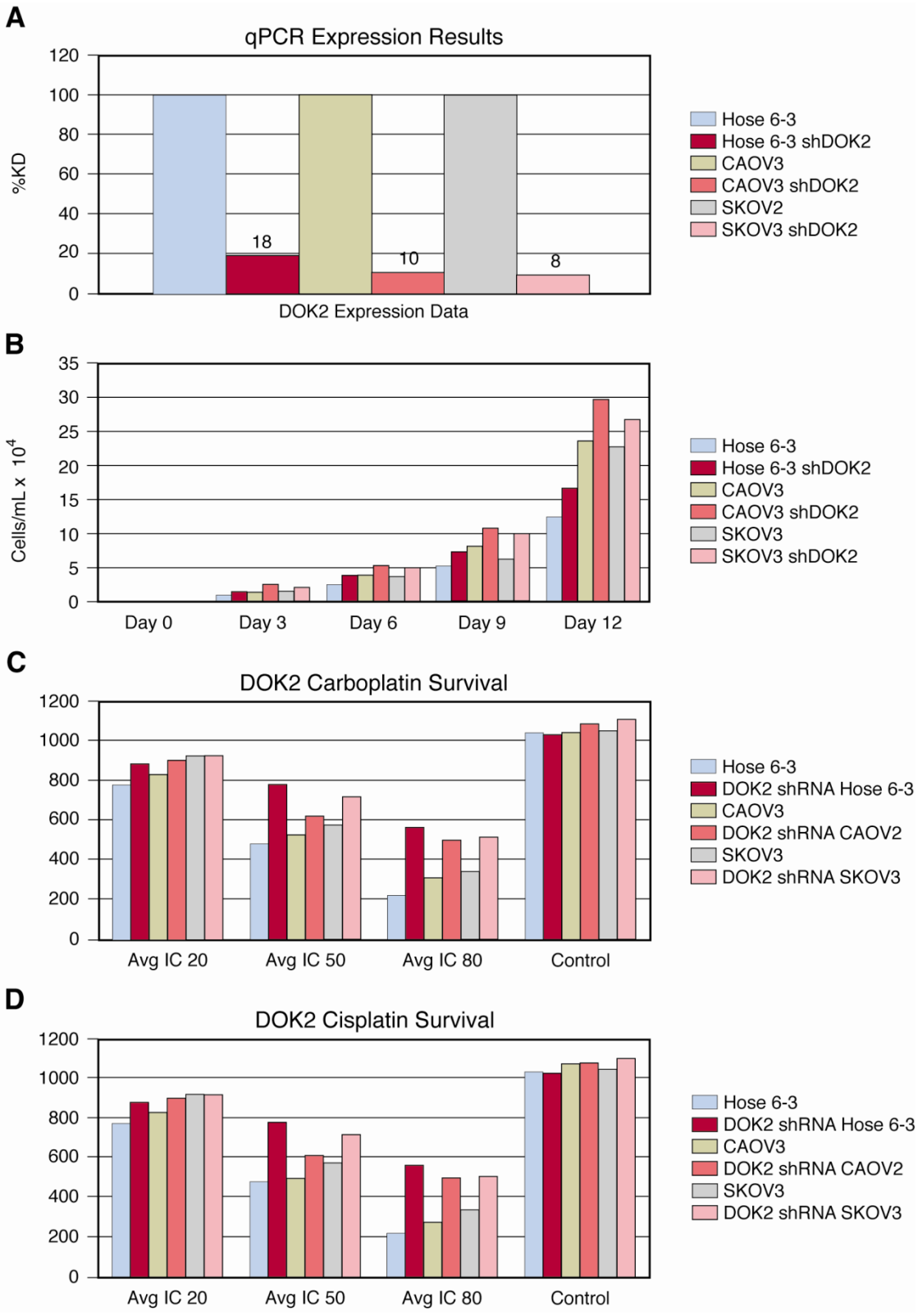
calculating the efficiency of shRNA pLKO.1 induced knockdown relative to the control non target shRNA infection. The Y axis is the percentage transcript with the non target shown as 100% by definition of being the reference. Each bar represents one of the infections in one of the three cell lines used. Inset on top of the bars for cell lines with *CHD3* shRNA is the level of transcript determined to be remaining after shRNA induced knockdown. **Panel B** is a graph MTT assay cell proliferation over a 12 day period. The x-axis is the time line and the y-axis is cell growth. As you can see, just like pGIPZ, the cells with the sh*CHD3* appear to be proliferating at a slower rate. **Panel C** shows carboplatin resistance as a bar graph that represents cell survival over a 3 cycle treatment of carboplatin at the varying doses of IC20, IC50, and IC80. Y-axis represents cell counts in cells/ml and the x-axis represents chemotherapy dose from IC20-IC80. Cell lines are listed on the right side of the panel. The bars shown are the means ± SD (n = 12) of triplicate measurements.

Appendix 2-5 Gene validation in ovarian tumors



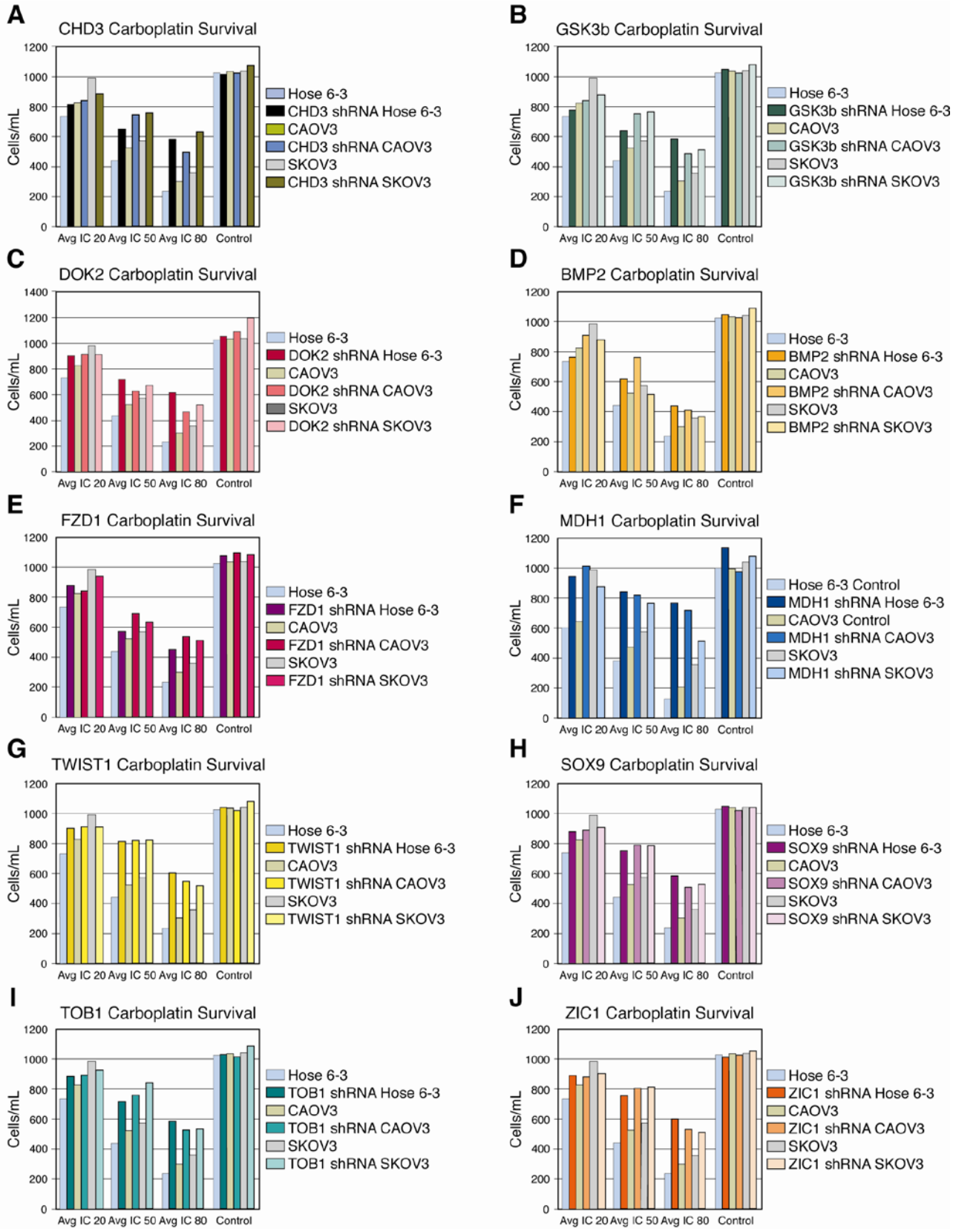
Appendix 2-5: This figure represents ovarian cancer tumor information on the validated genes from the functional screen. Green indicates a mutation, red indicates amplification, blue represents homozygous deletion, and gray indicates down-regulation. This is publically available data from the Cancer Genome Atlas.

Appendix 2-6 DOK2 Experiments



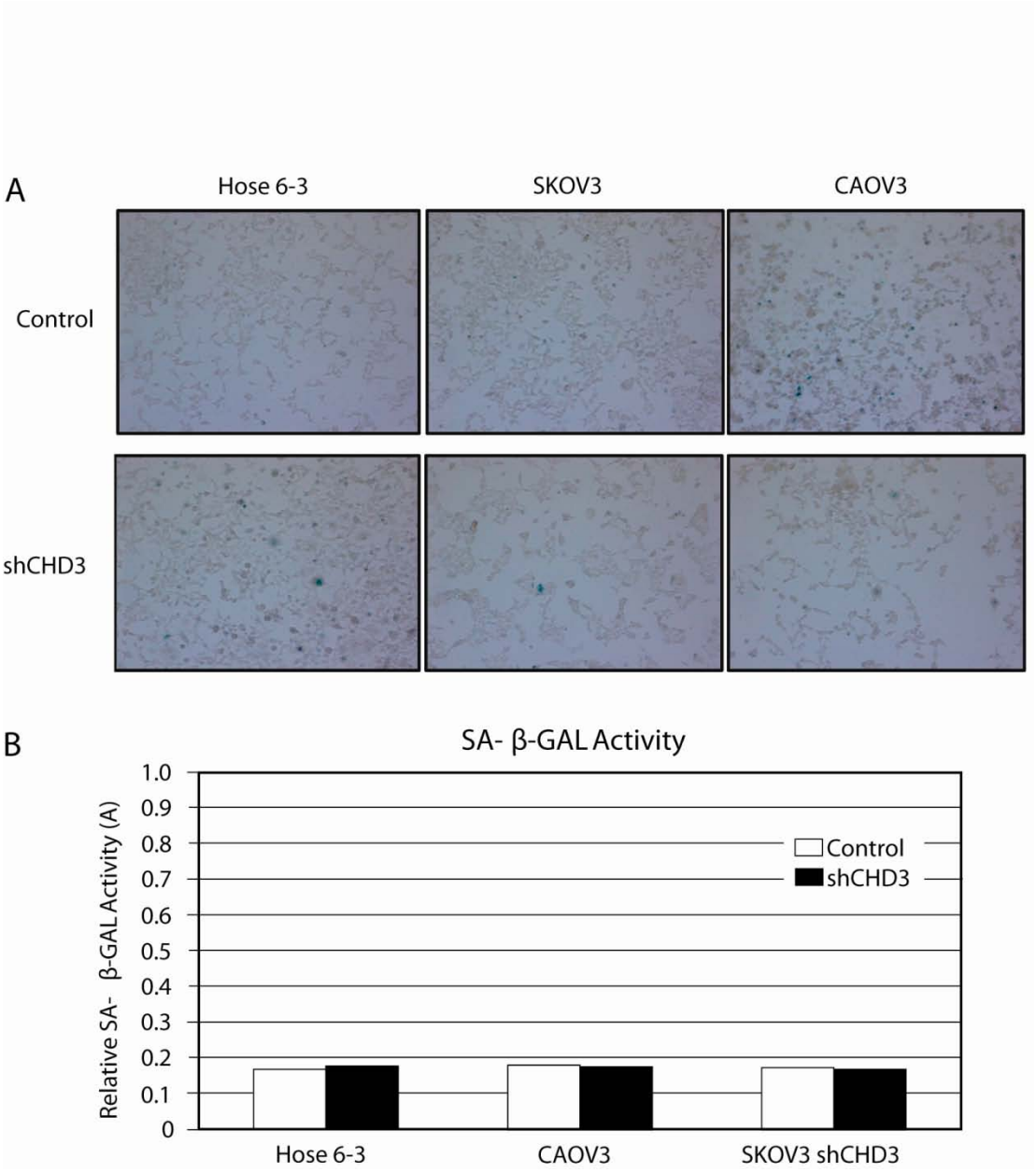
Appendix 2-6: Represents the chemoresistance experiments with the shRNA to DOK2, another candidate from the functional validation in vitro screen. Although the dissertation research did not focus on this candidate, it still validated in several of the assays applied. **Panel A** shows validation of specificity of the pGIPZ shRNA clones targeting *DOK2*. This graph represents a bar plot of RT-Q-PCR analysis of the shRNA infections calculating the efficiency of shRNA pGIPZ induced knockdown relative to the control non target shRNA infection. The Y axis is the percentage transcript with the non target shown as 100% by definition of being the reference. Each bar represents one of the infections in one of the three cell lines used. Inset on top of the bars for cell lines with *DOK2* shRNA is the level of transcript determined to be remaining after shRNA induced knockdown. **Panel B** represents MTT assay cell proliferation over a 12 day period. The x-axis is the time line and the y-axis is cell growth. **Panel C-D** shows platinum resistance as a bar graph that represents cell survival over a 3 cycle treatment of drugs at the varying doses of IC20, IC50, and IC80. Y-axis represents cell counts in cells/ml and the x-axis represents chemotherapy dose from IC20-IC80. Cell lines are listed on the right side of the panel. The bars shown are the means ± SD (n = 12) of triplicate measurements.

Appendix 3 Carboplatin Survival Curves for Validated Genes



Appendix 3: Illustrate bar graphs that represent cell survival over a 3 cycle treatment of carboplatin drugs at the varying doses of IC20, IC50, and IC80. Y-axis represents cell counts in cells/ml and the x-axis represents chemotherapy dose from IC20-IC80. Cell lines are listed on the left side of the panel. Gene names are listed on the top of each graph. Each experiment was done in triplicate 96-well format. The bars shown are the means ± SD (n = 12, $P<0.06$).

Appendix 4 pLKO *CHD3* Does Not Cause a Significant Change in Senescence

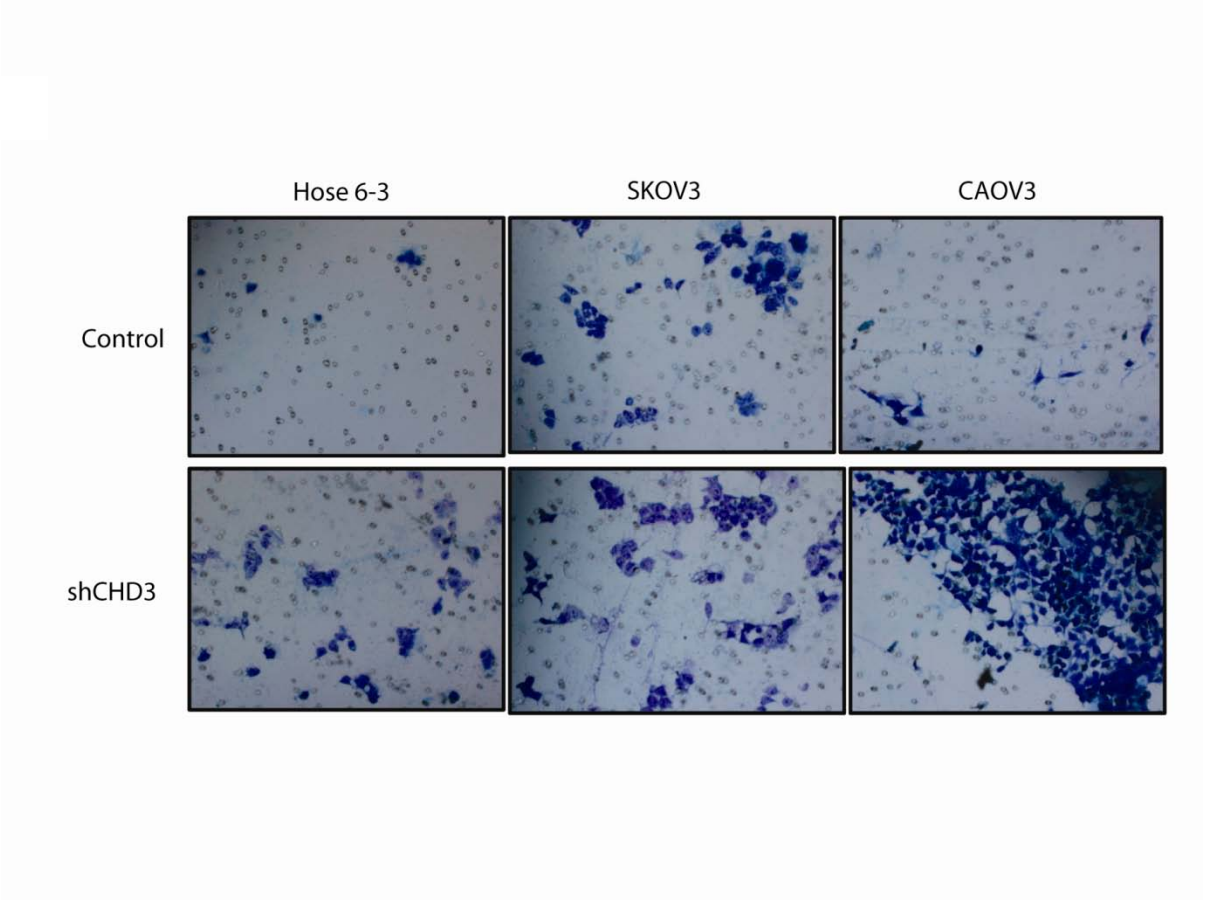


Appendix 4: Platinum-resistant *CHD3* cells do not cause a significant increase in senescence. **Panel A** represents SA-β-gal staining of the EOC cells with and with sh*CHD3* (Scale bar, 100 μm.). Top panels represent the control cell lines and the bottom panels represent shRNA infections with *CHD3*. **Panel B** is a bar graph of the results

relative % senescence-associated β-galactosidase (SA-β-gal) staining in the cell populations. The y-axis represents the relative % senescence-associated β-galactosidase activity and the x-axis represents the EOC cell lines with and without the shRNA to *CHD3*. Averages were plotted from at least 6 fields of view under a phase contrast microscope.

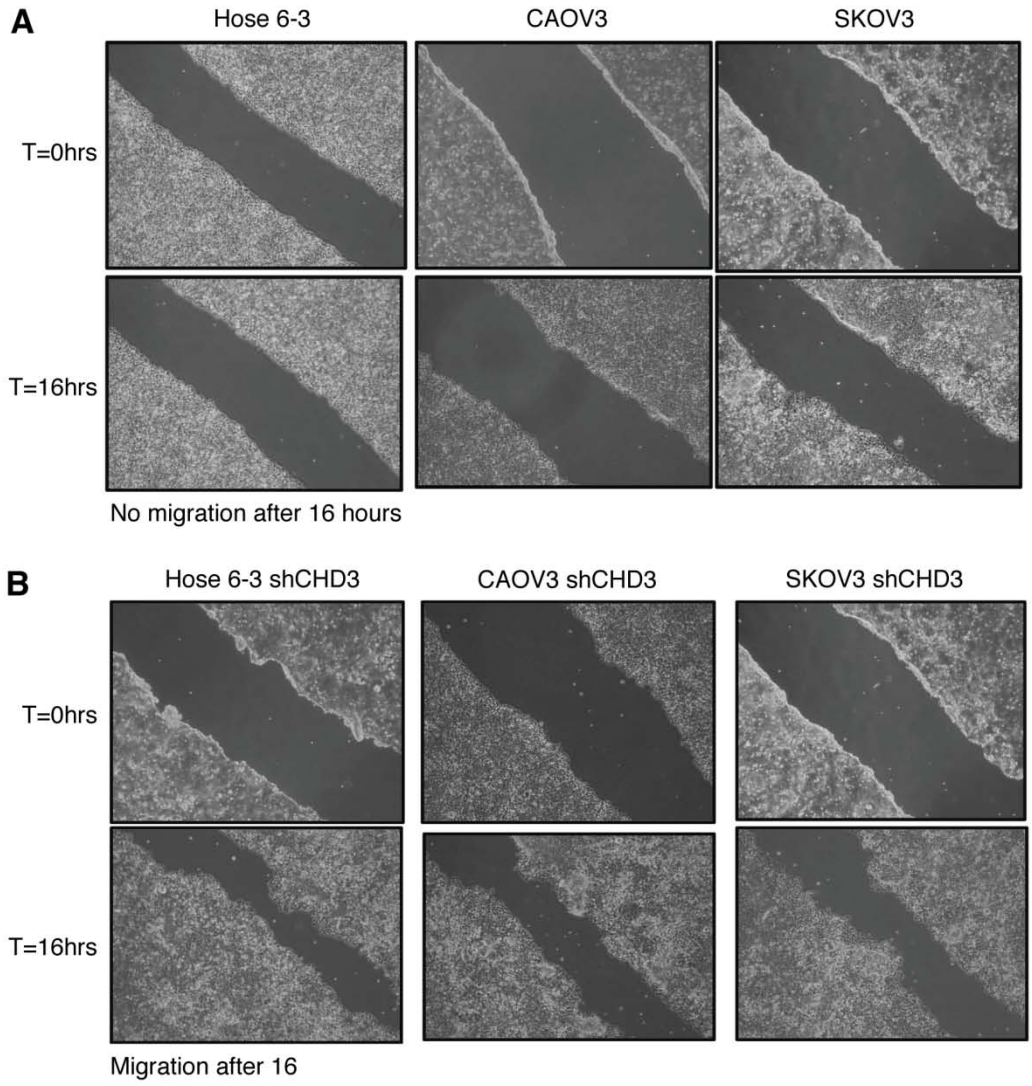
Appendix 5 pLKO sh*CHD3* Confers Invasion, Migration, and EMT

Appendix 5-1 Invasion Analysis for pLKO *CHD3* shRNA



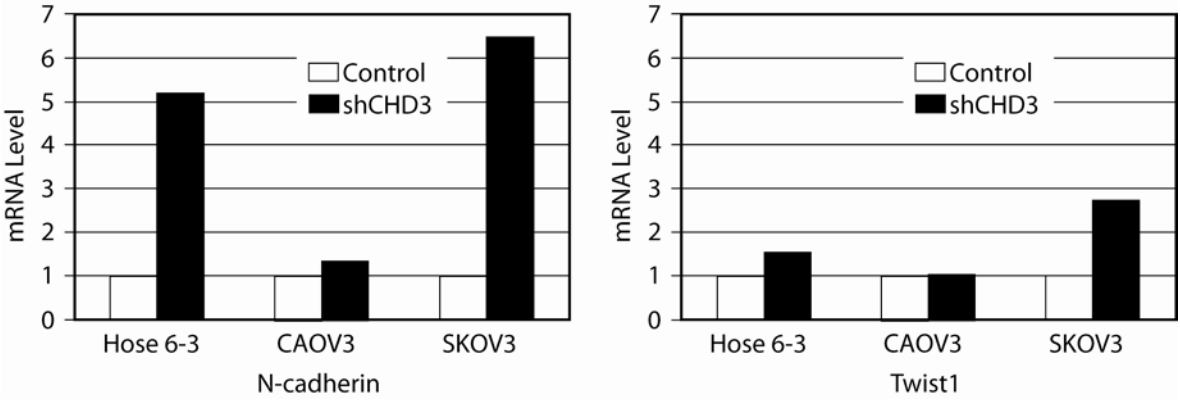
Appendix 5-1: Platinum-resistant pLKO *CHD3* cells are characterized by increased invasion metastatic migration potential. Suppression of *CHD3* markedly increased invasion ability as measured by invasion assay.

Appendix 5-2 Migration Analysis for pLKO CHD3



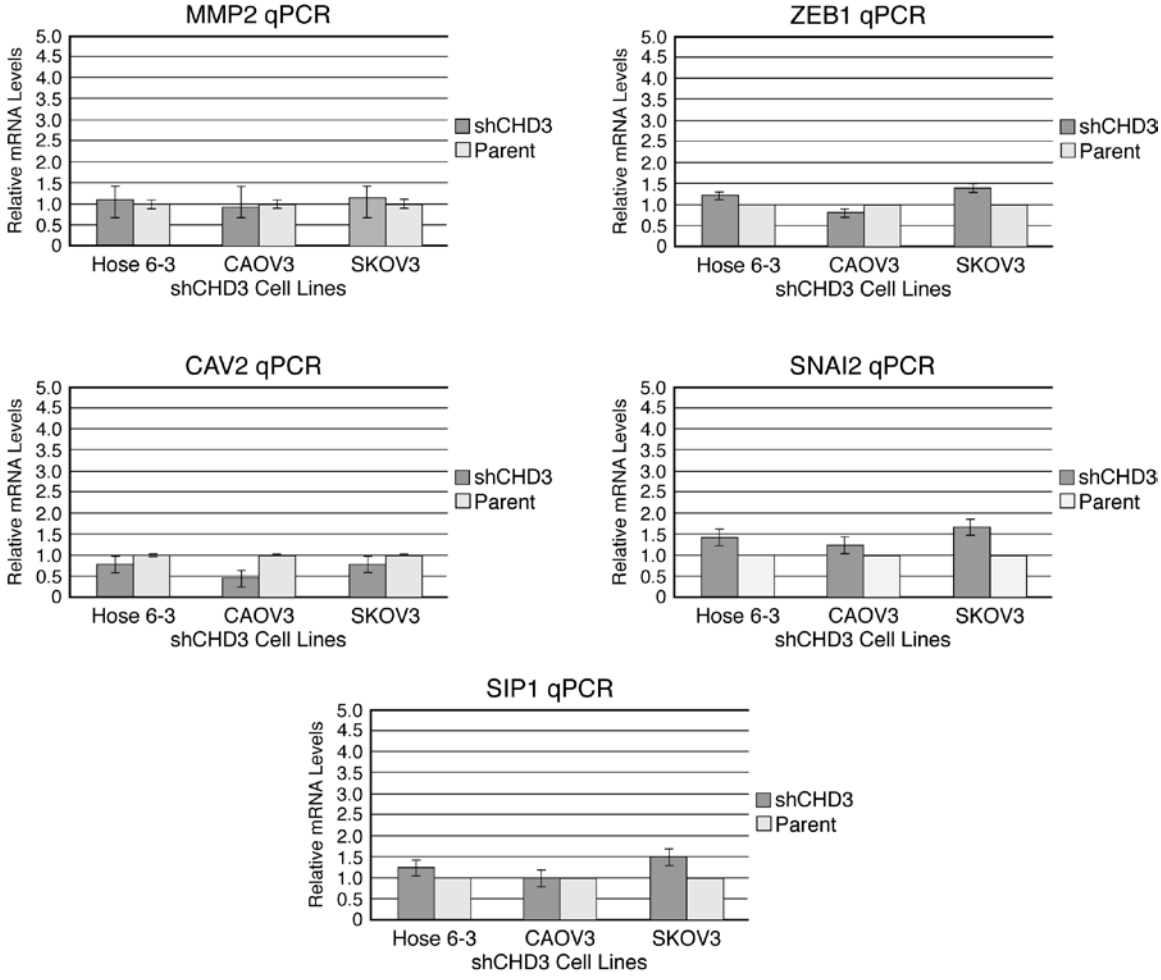
Appendix 5-2: Panel A-B Platinum-resistant pLKO *CHD3* cells are characterized by metastatic migration potential. **Panel A-B** are wound healing assays that represents cells migrating to heal a scratch immediately after scratching the cell monolayer (time = 0 h) and after 12 h. Cell images were taken under phase-contrast microscopy. Images from the controls (Hose 6-3, CAOV3, SKOV3) are represented in panel A and sh*CHD3* infected cells are represented in panel B. **Panel C** represents the quantification of the migration depicted as a change in area (%). Error bars represent the standard deviation (n=2). The X axis are the cell lines and the Y axis is the % migration comparison from the 0 time point and after 16 hours.

Appendix 5-3 pLKO *CHD3* RT-Q-PCR for EMT



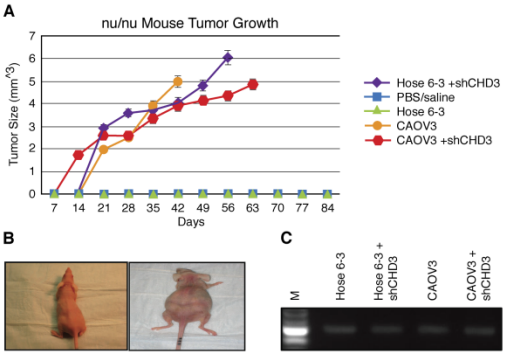
Appendix 5-3: Knock-down of *CHD3* Regulates EMT Associated Markers. The bar plots represent RT-Q-PCR analysis of the shRNA infections calculating the transcript level of EMT markers N-cadherin (left) and Twist1 (right) in control cell lines and sh*CHD3* infected cell lines. The Y axis is the normalized message level with the non target shown as 1 by definition of being the reference. Each bar represents one of the infections in one of the three cell lines used.

Appendix 5-4 pGIPZ shCHD3 RT-Q-PCR EMT markers



Appendix 5-4 Knock-down of *CHD3* Regulates EMT Associated Markers. These bar graphs represent RT-Q-PCR of several EMT markers from the parental cell lines and shCHD3 silenced cell lines. The Y axis is the normalized message level with the non target shown as 1 by definition of being the reference. Each bar represents one of the infections in one of the three cell lines used. The error bars represent standard deviations from 2 independent experiments (n=3, $P<0.06$).

Appendix 5-5 pGIPZ shCHD3 Nude Mice Survival Analysis



Appendix 5-5 illustrates growth of the parental cells and cells with the shCHD3 in nude mice over several weeks. **Panel A** is tumor growth in Hose 6-3 and CAOV3 cells lines. As you can see from the graph, tumors formed within one week of intraperitoneal cell injections. However, in both the Hose 6-3 cell parental cell line as well as the PBS/Saline control, there was no tumor growth as expected. Error bars represent three independent experiments (n=3, $P<0.05$). **Panel B** shows a picture of the nude mice. The left image represents the PBS/saline i.p. injected mouse and the right image represents Hose 6-3 with the shCHD3 i.p. injected mouse. The right mouse has several i.p. tumors. Panel C is an ethidium bromide stained gel demonstrating that DNA from the tumors of nude mice can be extracted and amplified using universal primers to show the incorportation of the shCHD3 insert in the tumors.