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E3 Ligase Identified by Differential Display (EDD) Enhances Cell Survival and Cisplatin Resistance in Epithelial Ovarian Cancer and Oral Squamous Cell Carcinoma

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A dissertation submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirement for the degree of Doctor of Philosophy in the college of Graduate Studies

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I dedicate this to my loving husband, Andy Bradley, for his patience and support. Without his love and understanding I could not have completed my work for this dissertation. You believed in me when I did not have the courage to believe in myself. You encouraged me to be persistent in my efforts. I know that you have spent many hours praying for my experiments, exams, presentations, and my sanity. I appreciate your notes and emails of encouragement. You always know how to make me laugh and relax after a long day in lab.

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List of Abbreviations

ACVRL1 - activin receptor-like kinase 1

Akt - protein kinase B (PKB)

AP-1 - Activator protein 1

APC - adenomatous polyposis coli

ATM - ataxia telangiectasia mutated

Bcl2 - B cell lymphoma-2

Bcl-xL - B cell lymphoma-x long

BRCA1/2 - Breast cancer type 1 or 2 susceptibility protein

β-TrCP - β-transducin repeat-containing protein

Chk2 - checkpoint kinase 2

DOPC - 1,2-dioleoyl-sn-glycero-3-phophatidylcholine

DYRK2 - dual specificity tyrosine phosphorylation regulated kinase 2

DUB - Deubiquitinating enzyme

EDD - E3-ubiquitin ligase identified by differential display

E2F1 - E2F transcription factor 1

ERK - Extracellular signal-regulated kinase

GATA-2 - GATA-binding protein 2

GFP - green florescent protein

GRHL2 - grainyhead-like 2

GSK3β - glycogen synthase kinase 3 beta

HECT - Homologous to the E6-AP carboxyl terminus

HER2 - Human epidermal growth factor receptor 2

HIF-1α - hypoxia-inducible factor 1 alpha subunit

HPV - human papillomavirus

HSF1/2 - Heat shock factor protein 1/2

Hyd - hyperplastic discs

JNK - Jun N-terminal protein kinase

KRAS - Kirsten Rat Sarcoma Viral Oncogene Homolog

MAPK - Mitogen-activated protein kinase

Mcl-1 - myeloid cell leukemia sequence 1

MEK/MKK - MAP (mitogen-activated protein) kinase kinase

NF-kappa B - Nuclear Factor Kappa Beta

p38 - p38 MAPK

p53 - tumor protein 53

PAK - p21 activated kinase

Paip2 - polyA-binding protein inhibitory protein 2

PARP - Poly(ADP-ribose)polymerase

PDGF - platelet-derived growth factor

PI3K - Phosphoinositide-3-Kinase

PR - progesterone receptor

Puma - p53 up-regulated modulator of apoptosis

RIP1 - receptor-interacting protein 1

SDS PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

SRB assay - Sulforhodamine B assay

TNFα - tumor necrosis factor α

TopBP1 - Topoisomerase Binding Protein 1

UBH - Ubiquitin carboxy-terminal hydrolases

UBP - ubiquitin-specific processing proteases

Ubr5 - ubiquitin protein ligase E3 component n-recognin 5

VEGF - vascular endothelial growth factor

Abstract

AMBER THOMPSON BRADLEY. E3 Ligase Identified by Differential Display (EDD) enhances cell survival and cisplatin resistance in epithelial ovarian cancer and oral squamous cell carcinoma. (Under the direction of Scott Eblen).

EDD (E3-ubiquitin ligase identified by Differential Display) is an E3 ubiquitin ligase that is overexpressed in ovarian cancer, but is rare in benign and borderline tumors. EDD is also overexpressed in recurrent, platinum-resistant ovarian cancers and is associated with a two-fold increased risk of disease recurrence and death in ovarian cancer patients, suggesting a role in tumor survival and/or platinum resistance. EDD knockdown by siRNA induced apoptosis in A2780ip2, OVCAR5, and ES-2 ovarian cancer cells, correlating with a loss of the anti-apoptotic protein Mcl-1 through a GSK-3β-independent mechanism. Transient knockdown of EDD or Mcl-1 induced comparable levels of apoptosis in A2780ip2 and ES-2 cells. Stable overexpression of Mcl-1 protected cells from apoptosis following EDD knockdown, accompanied by a loss of endogenous, but not exogenous, Mcl-1 protein, indicating that EDD may regulate Mcl-1 synthesis. Indeed, EDD knockdown induced a 1.87-fold decrease in Mcl-1 mRNA and EDD transfection enhanced murine Mcl-1 promoter driven luciferase expression five-fold. To separate EDD survival and potential cisplatin resistance functions, we generated EDD shRNA stable cell lines that could survive initial EDD knockdown and demonstrated that these cells were four- to 21-fold more

sensitive to cisplatin. Moreover, transient EDD overexpression in COS-7 cells was sufficient to promote cisplatin resistance 2.4-fold, dependent upon its E3 ligase activity. *In vivo*, mouse intraperitoneal ES-2 and A2780ip2 xenograft experiments showed that mice treated with EDD siRNA by nanoliposomal delivery (DOPC) along with cisplatin had significantly less tumor burden than those treated with control siRNA/DOPC alone (ES-2, 77.9% reduction, p=0.004; A2780ip2, 75.9% reduction, p=0.042) or control siRNA/DOPC with cisplatin in ES-2 (64.4% reduction, p=0.035), with a trend in A2780ip2 (60.3% reduction, p=0.168). These results identify EDD as a dual regulator of cell survival and cisplatin resistance and suggest EDD is a therapeutic target for ovarian cancer. Additionally, *edd* is overamplified in oral squamous cell carcinoma of the tongue. Preliminary results in this carcinoma indicate similar roles of EDD in regulating cellular survival and cisplatin resistance as demonstrated in ovarian cancer.

Chapter 1

Introduction

Ovarian Cancer

Ovarian cancer is the leading gynecological cancer and the fifth leading cause of cancerous deaths of females in the United States. Approximately 20,000 women each year are diagnosed with this cancer with about 14,000 women dying each year from this disease (Siegel, Naishadham et al. 2013). Most women will die from recurrence after their tumors are drug resistant. The death rate is high in ovarian cancer patients because symptoms are overlooked or patients are not diagnosed until the disease has advanced. Common symptoms such as abdominal bloating and pain are not present until the tumors have metastasized throughout the peritoneal cavity. Current conventional treatments include surgical debulking of the tumor or removal of the reproductive organs, along with chemotherapy treatment such as paclitaxel, cisplatin, and/or carboplatin.

Etiology of Epithelial Ovarian Cancer

Over 85% of ovarian tumors are epithelial carcinomas, which are thought to arise from the ovarian surface epithelium (mesothelium) (Auersperg, Wong et al. 2001). Tumors can also develop out of germ cells or stromal cells, which maintain the structural integrity of the ovaries and supply hormones. The origin of ovarian cancer has been highly debated over the past decade. Research in patients with familial ovarian cancer, arising as a result of mutations in BRCA1,

BRCA2, or p53, has indicated that these cancers may originate from the fallopian tubes (Selvaggi 2000, Powell, Kenley et al. 2005, Medeiros, Muto et al. 2006, Lee, Miron et al. 2007, Mehrad, Ning et al. 2010, Kurman and Shih Ie 2011). More recent research has also linked the fallopian tubes to the origin of ovarian cancer in non-hereditary cases (Lee, Miron et al. 2007, Mehrad, Ning et al. 2010, Kurman and Shih Ie 2011). Most epithelial ovarian tumor cells resemble cells from the fimbria, the distal part of the fallopian tube. It is difficult to determine the etiology of ovarian carcinoma because patients are not usually diagnosed until they exhibit advanced stages of the disease. This is a result of the dismissal of common symptoms of ovarian cancer such as abdominal pressure, bloating, pelvic pain, nausea, constipation, loss of appetite, and loss of energy. These symptoms are vague and often associated with more common issues such as digestive problems, leading to their dismissal as symptoms of ovarian cancer. Left untreated, ovarian cancer typically spreads locally to the opposite ovary, uterus, and the intraperitoneal cavity. While rare, metastasis to the liver, adrenal glands, spleen, and lungs may occur in the most aggressive forms of cancer. The most prevalent form of ovarian cancer is serous epithelial cancer (Seidman, Horkayne-Szakaly et al. 2004). It is commonly believed now that serous ovarian cancer originates from the fallopian tube. Other epithelial ovarian carcinomas can be classified as mucinous, endometrioid, clear cell, transitional cell (Brenner type), squamous, and mixed epithelial.

Risk Factors

The most common risk factors for ovarian cancer are heredity. Other risk factors include hormonal and environmental sources. The tumor protein 53 (p53) is commonly mutated in many types of cancers, including ovarian cancer. The tumor suppressor p53 activates DNA damage repair when damage is sensed, resulting in cell cycle arrest until the damage is repaired and apoptosis if the damage is irreparable. Alterations in p53 exist in 96% of high grade ovarian serous carcinomas, the most common subtype of ovarian cancer, but are rare in low grade serous carcinomas (Green, Berns et al. 2006). Human epidermal growth factor receptor 2 (HER2) is overexpressed in many types of cancers as well and is associated with a poor prognosis in these patients. HER2 is a receptor in the ErbB family of receptors and this protein regulates the signaling pathways linked to promoting cell proliferation and prevention of apoptosis. Overexpression of HER2 is estimated to be present in 10% of ovarian cancers (Verri, Guglielmini et al. 2005). Mutations in BRCA1 and BRCA2 in patients indicate a 30% to 70% chance of developing ovarian cancer by the age of 70 (Antoniou, Pharoah et al. 2003, Chen and Parmigiani 2007). BRCA proteins are involved in mismatch repair mechanisms to repair DNA damage in the double helix such as during homologous recombination. Alterations in the cyclin kinase inhibitors p21 and p27, and the cell cycle protein cyclin E (Bali, O'Brien et al. 2004, Schmider-Ross, Pirsig et al. 2006, Nakayama, Nakayama et al. 2010) are also present in ovarian tumors. Alterations in other signaling pathways can also

occur as a result of mutations in KRAS (Vereczkey, Serester et al. 2011) and PI3 kinase (p110 subunit) (Levine, Bogomolniy et al. 2005).

Other risk factors related to the development of ovarian cancer include environmental and hormonal factors. These risks are related to the commonly believed predisposition to ovarian cancer – ovulation (Fathalla 1971). It has been a long held belief that ovarian cancer arises as a result of the disruption and repair of the epithelial cells in the ovary due to ovulation and the oocyte leaving the ruptured follicle. Supporting this theory, pregnancy and the use of oral contraceptives to regulate the ovulation cycle are well known to reduce the risks of developing ovarian cancer. Ovarian cancer typically affects menopausal and post-menopausal women. Infertility has been shown to increase the risk of ovarian cancer, but it is unclear whether this is due to the lack of pregnancy or the use of fertility drugs which promotes this effect. Since hormones control the ovulation cycle, gonadotropins (Cramer and Welch 1983), which stimulate the ovarian epithelium, and estrogens and androgens, which promote reproductive capabilities, are known to promote carcinogenesis (Parazzini, La Vecchia et al. 1994, Karlan, Jones et al. 1995, Rodriguez, Calle et al. 1995, Silva, Tornos et al. 1997). In the 1960s, an association was found between the use of talcum powder and an increased risk of ovarian cancer, which suggests that toxins can enter the genital track and migrate upward to the reproductive organs (Henderson, Hamilton et al. 1979, Harlow, Cramer et al. 1992, Huncharek, Geschwind et al. 2003).

Treatment of Ovarian Cancer

Standard treatment of ovarian cancer is a combination of cytoreductive surgery and chemotherapies such as taxane (paclitaxel) and platinum (cisplatin or carboplatin) drugs. These treatments have been the standard of care for ovarian cancer patients for the last few decades, indicating a need for updated strategies. Surgical cytoreduction may include a total hysterectomy, bilateral salpingo-oophoectomy (removal of the ovaries and fallopian tubes), removal of pelvic or para-aortic lymph nodes, and/or omentectomy (removal of the abdominal lining) (Kim, Ueda et al. 2012). Clear cell carcinomas are notoriously resistant to paclitaxel and carboplatin, so these tumors are typically treated with irinotecan and cisplatin. Chemotherapies that are under investigation are PARP inhibitors and bevacizumab, an antibody directed against vascular endothelial growth factor (VEGF). PARP inhibitors show promising results in patients with BRCA mutations, since both PARP and BRCA are involved in DNA damage repair.

Following drug activation by aqueous hydrolysis, cisplatin and carboplatin exert their activity through interaction with DNA, RNA, and protein with a cytotoxic effect mediated through the formation of interstrand and intrastand crosslinks in DNA, creating DNA adducts. This elicits a DNA damage response in the cell, where nucleotide excision repair and mismatch repair are able to repair the damage. If the damage is not repaired, then the DNA damage signals for apoptosis to occur. Cisplatin (*cis*-diammine dichloroplatinum (II)) was first discovered in 1965 as an inhibitor of proliferation by Rosenberg and colleagues

when studying *Escherichia coli* exposed to a current delivered by platinum electrodes (Rosenberg, Vancamp et al. 1965). It was discovered that a platinum complex, specifically the *cis* isomer, inhibited binary fusion in the bacteria. Carboplatin (*cis*-diammine (1,1-cyclobutanedicarboxylate) platinum (II)) was created as an analog to cisplatin to reduce severe side effects, such as nephrotoxicity (kidney damage), neurotoxicity (nerve damage), and ototoxicity (hearing loss). In clinical trials, carboplatin has the same efficacy as cisplatin in ovarian cancer, although cisplatin is still more effective in other types of cancer, such as head and neck cancer. Paclitaxel was first discovered and isolated from the bark of the Pacific yew tree (*Taxus brevifolia*) in 1967 (Wani, Taylor et al. 1971). Paclitaxel exerts its effect through the stabilization of microtubules to ultimately prevent breakdown of microtubules during mitosis.

Recurrent Ovarian Cancer

The five year survival rate of ovarian cancer patients is low (35%) after treatment with surgical debulking and chemotherapy treatment (paclitaxel, carboplatin, and/or cisplatin) (Green, Berns et al. 2006). Initial response rates to surgery and chemotherapy is 70%-80% in ovarian cancer patients (du Bois, Luck et al. 2003, Ozols, Bundy et al. 2003). However, most patients will eventually relapse with a poor prognosis and progression-free survival time of only 18 months in those with advanced disease (McGuire, Hoskins et al. 1996, Rubin, Randall et al. 1999). There are a multitude of proposed mechanisms of resistance to both platinum drugs and paclitaxel. Many mechanisms overlap as major mechanisms of resistance to various chemotherapeutic drugs (Siddik

2003). Table 1.1 contains a list of identified mechanisms of resistance to the platinum drugs and paclitaxel, although this table provides a selective number of mechanisms and is not meant to be an exhaustive description of mechanisms of resistance to these chemotherapies. Most methods of resistance are relevant to the mechanism of action of each drug, as described above, or its presence in the cell. Obviously, it is extremely difficult to target the potential cellular methods of resistance to these drugs, indicating the need for better therapeutics and/or chemotherapeutic drug combinations in an effort to decrease multidrug resistance in tumors.

Table 1.1

General Mechanisms of Resistance to Platinum Chemotherapy or Paclitaxel

Resistance Mechanisms to Platinum Drugs	**Resistance Mechanisms to Paclitaxel**
Increased Efflux Drug Transporters (ABC, MDR transporters)	
Decreased Uptake Drug Transporters	
Loss of p53 Function	
Downregulation of Pro-apoptotic proteins (Bax, Bad)	
Upregulation of Anti-apoptotic proteins (Bcl-2, Bcl-XL, Mcl-2)	
Increased Drug Inactivation (Glutathione and metallothioneins)	
Increased DNA damage repair	Modifications of Tubulin
Increased MAPK pathway	Delay in mitotic entry
Ras mutation or overexpression	
HER2 overexpression	
Increased PI3K/Akt pathway	

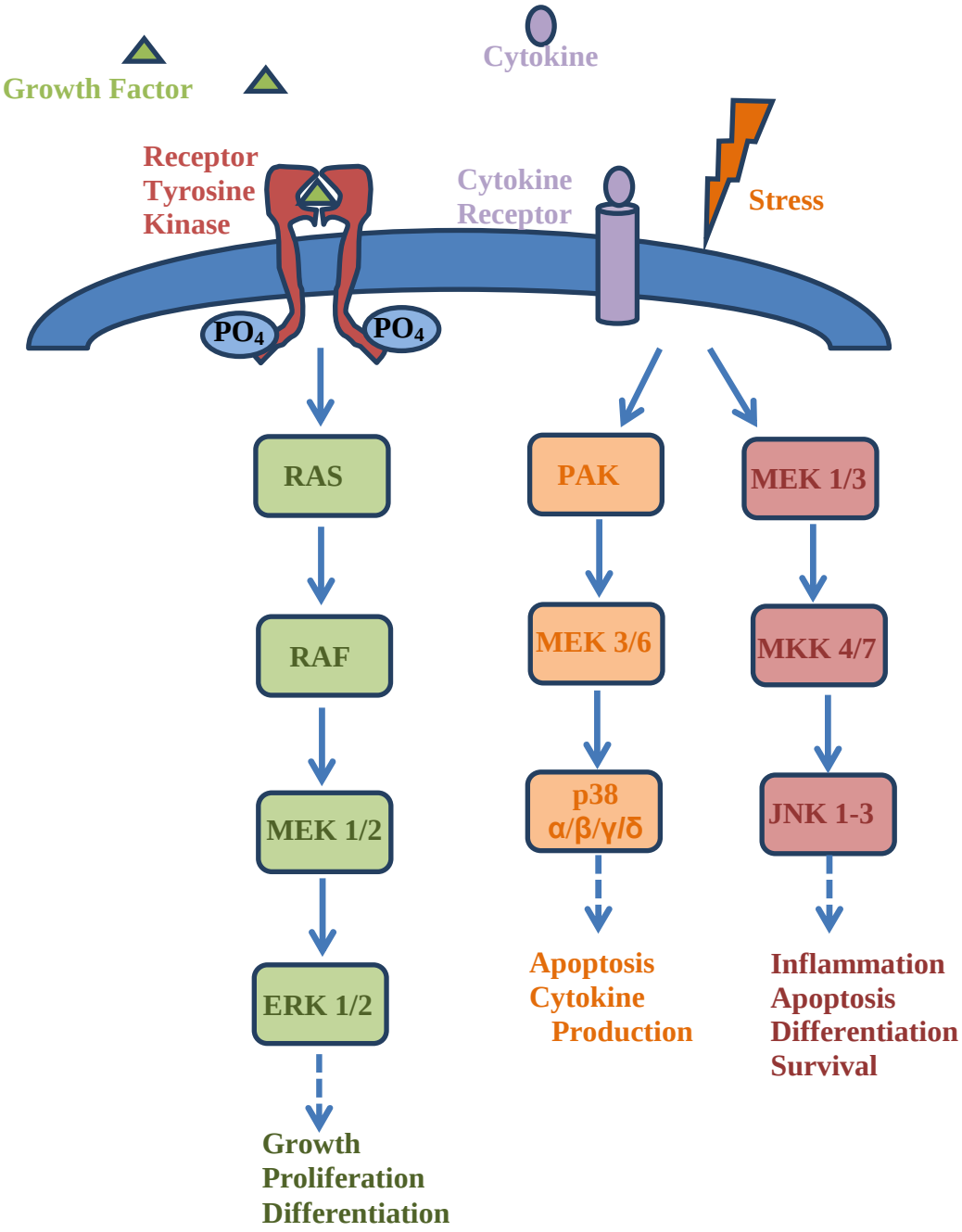
MAP Kinase Signaling in Ovarian Cancer

Activating mutations of BRAF and KRAS are prevalent in low grade and borderline ovarian tumors (approximately 60%), but is rarely observed in high grade serous ovarian carcinomas (Singer, Oldt et al. 2003). These mutations are mutually exclusive. This indicates that activation of signaling through RAF and

RAS are important in the development of ovarian tumors. Downstream of RAF and RAS, the activation of the MAP (mitogen-activated protein) kinase pathway has been detected in 80% of low grade ovarian tumors and 40% of high grade tumors (Hsu, Bristow et al. 2004). The MAPK pathway is critical for the transmission of extracellular signals, such as a response to the presence of growth factors, stress, and cytokines, to intracellular signaling pathways. Activation of the MAPK pathway leads to the activation of protein kinases, transcription factors, and other nuclear proteins which can lead to extensive proliferation and evasion of apoptosis, promoting tumor development. Constitutive activation of the MAPK pathway also leads to alterations in other pathways, such as the Akt pathway, which also participates in the development of tumors.

The MAPK pathway includes three major pathways which are differentially regulated by extracellular signals and thus lead to unique intracellular signaling processes. This is illustrated in Figure 1.1 which depicts an extremely simplified view of MAPK signaling pathways. There are many other upstream and downstream proteins involved in each signaling cascade as well as considerable crosstalk between these pathways and other pathways not included in this figure.

Figure 1.1. MAP Kinase Signaling Pathways


Growth Factor
Cytokine
Receptor
Tyrosine
Kinase
Cytokine
Receptor
Stress
PO4
PO4
RAS
RAF
MEK 1/2
ERK 1/2
Growth
Proliferation
Differentiation
PAK
MEK 3/6
p38
α/β/γ/δ
Apoptosis
Cytokine
Production
MEK 1/3
MKK 4/7
JNK 1-3
Inflammation
Apoptosis
Differentiation
Survival

Oral Squamous Cell Carcinoma

Approximately 41,380 Americans each year are diagnosed with cancers of the oral cavity or pharynx, affecting more males (29,620) than females (11,760) (Siegel, Naishadham et al. 2013). Almost 20% of Americans that are diagnosed with carcinoma affecting these tissues will die from this disease each year. Worldwide, cancers of the oral cavity and pharynx affect 337,931 people each year, with a rate of mortality of approximately 54% (Parkin, Bray et al. 2005). The five year survival in the United States has increased to about 65% (increased from 53% in the 1970s) (Siegel, Naishadham et al. 2013). In contrast, the five year survival in developing countries for these patients is only about 31% (Parkin, Bray et al. 2005). Diagnosis typically occurs in patients 50 years old or older.

Etiology

Squamous cell carcinomas of the oral cavity can affect the tongue, base of the tongue, tonsils, nasopharynx, pharynx, and larynx (Saba, Goodman et al. 2011). Each of these carcinomas is distinct. This type of carcinoma affects squamous cells, a type of epithelial cell. Molecular changes in these cells leading to carcinoma include gene amplification or overexpression of oncogenes such as *erbB2*, *epidermal growth factor receptor (EGFR)*, *myc*, and *cyclin D1*or mutations in tumor suppressors such as *p53* or *p16* (Mehrotra and Yadav 2006).

Risk Factors

The high prevalence of oral cancer around the world, especially in developing countries, is due to increased use of tobacco (either chewing or

smoking) and alcohol use, the primary causes of oral cancer. Viral infection with Human papillomavirus (HPV), commonly HPV 16 or HPV 18, also increases the risk of oral cancer while infection with Epstein-Barr virus has been linked to cancers of the nasopharynx (Parkin, Bray et al. 2005). Often HPV infection occurs as a result of oral-genital contact. Tumors linked to these viruses are often easier to treat allowing for increased survival in these patients (Ang, Harris et al. 2010). Typically, cancers of the tongue are not related to HPV infection, but rather to tobacco and alcohol use. Radiation exposure and immune deficiency have also been implicated in the development of these carcinomas.

Treatment of Oral Squamous Cell Carcinoma

Initially most oral carcinomas are asymptomatic often leading to a late diagnosis. Oral screenings from a dentist are critical to the diagnosis of this disease before progression and metastasis occurs. Treatments for oral squamous cell carcinomas of the various oral regions include surgical removal of the tumor(s), radiation, and chemotherapies such as cisplatin, paclitaxel, and 5-fluorouracil. Surgery and radiation are first line treatments with chemotherapy being used as an adjuvant therapy in patients with metastasis. Metastasis to the lymph nodes makes treatment much more difficult and decreases survival in these patients.

Mechanisms of Resistance

As with other cancers, resistance to chemotherapy treatment is a common problem in oral squamous cell carcinoma, exacerbated by the fact that chemotherapy is typically not used as an intervention until the disease has

progressed to the lymph nodes. Cancers affecting the oral cavity are unique in that the environment of the oral cavity has increased acidity. This increased acidity can affect the absorption of chemotherapeutic agents in these tumor cells (Griffiths 1991). This unique environment is thought to be maintained by vacuolar ATPases in these epithelial cells (Newell, Franchi et al. 1993, Yamagata, Hasuda et al. 1998). Other causes of drug resistance in these cells are common causes such as the overexpression of multi-drug resistance proteins to export drug out of the cell before allowing a cellular effect, as well as effects on the cell cycle, apoptosis, drug inactivation, and alterations in critical cellular pathways such as ERK, Akt, and p53. These common resistance mechanisms are described in more detail in the previous section on mechanisms of resistance in ovarian cancer.

Ubiquitin/Proteasome Pathway

History

The Nobel Prize in chemistry was awarded to Avram Hershko, Aaron Ciechanover, and Irwin Rose in 2004 for their work in the late 1970s to early 1980s discovering the degradation of proteins by ubiquitination. Hershko and colleges used fractionation of cellular components of reticulocytes to purify and identify proteins involved in ATP dependent protein degradation (Ciechanover, Heller et al. 1980, Hershko, Ciechanover et al. 1980, Hershko, Eytan et al. 1982, Hershko, Heller et al. 1983, Ciechanover, Hod et al. 2012).

Ubiquitin

The 76 amino acid polypeptide ubiquitin is expressed in all eukaryotes and is highly conserved. Ubiquitin is encoded by multiple genes, often as an oligomer, and processed to monomeric forms in order to be activated and covalently linked to proteins. The addition of ubiquitin to proteins is generally associated with protein degradation by the 26S proteasome (Hough, Pratt et al. 1986); however, dependent on the type of ubiquitin attachment, this may not always be the case. When a protein is labeled with a multi-ubiquitin chain by isopeptide bonds on Lys 6 (Nishikawa, Ooka et al. 2004), Lys 11, Lys 27, Lys 29 (Chastagner, Israel et al. 2006), Lys 33 (Al-Hakim, Zagorska et al. 2008), or Lys 63 (Deng, Wang et al. 2000, Wang, Deng et al. 2001, Herman-Bachinsky, Ryoo et al. 2007) of ubiquitin, this can regulate cellular activities independent of protein degradation, but these ubiquitin modifications can also signal for proteolysis. The initial ubiquitin is added to either the ε-amino group of lysine or the amino

terminal residue in the targeted protein (Ciechanover and Ben-Saadon 2004). Addition of a multi-ubiquitin chain on Lys 48 on ubiquitin is the prototypical ubiquitin modification on a protein to designate its degradation by the 26S proteasome. Monoubiquitination of proteins can regulate cellular functions or localization of the ubiquitylated protein (Levkowitz, Waterman et al. 1999, Mukhopadhyay and Riezman 2007). Ubiquitin-mediated cellular activities, independent of the proteasome, include kinase activation, transcription factor activation, protein translocation, endocytosis, lysosomal targeting, and DNA damage repair.

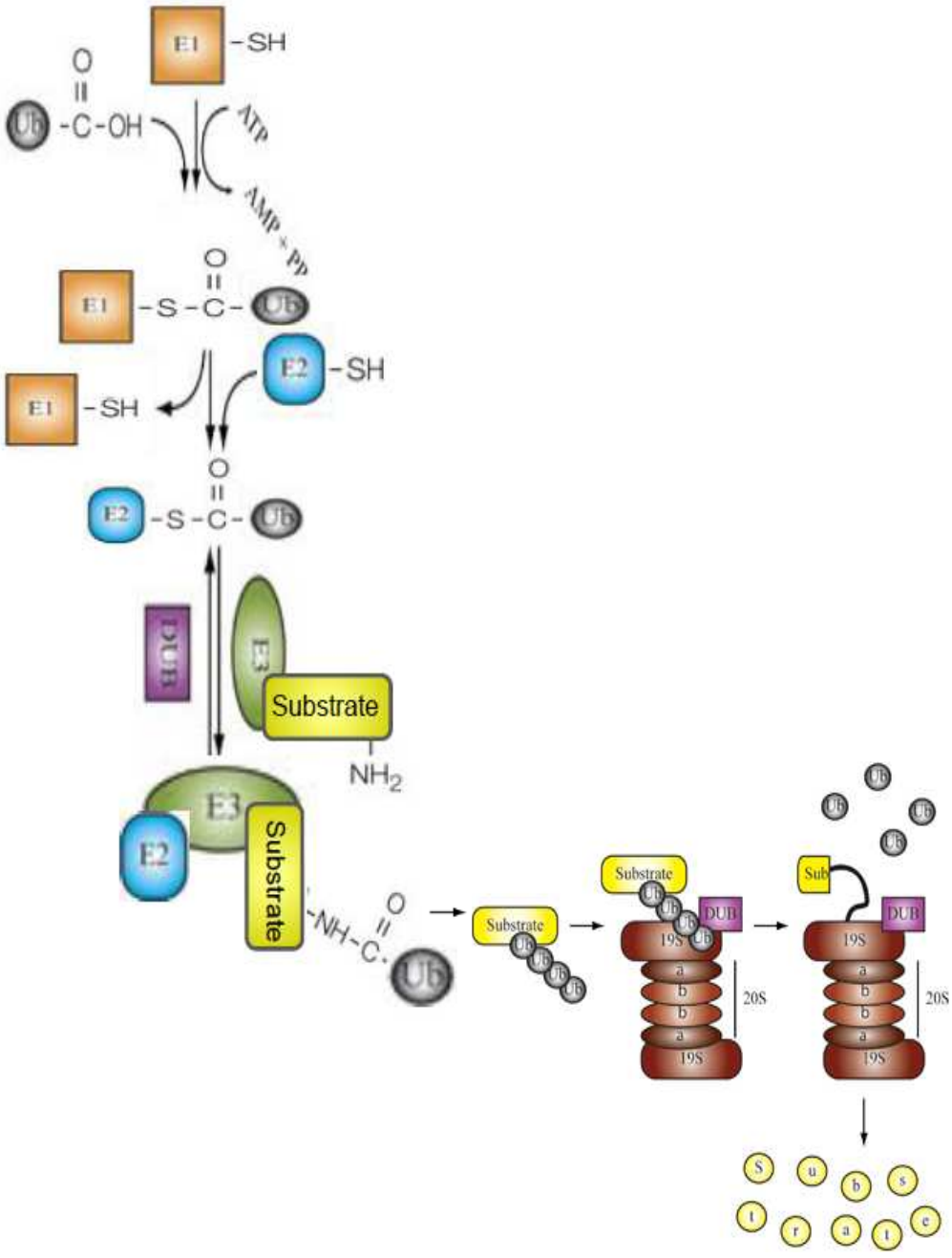
Ubiquitination

Ubiquitin activating E1 enzymes begin the ubiquitination pathway by binding to both MgATP and ubiquitin in order to form an activated ubiquitin adenylate (Haas and Rose 1982, Hershko, Heller et al. 1983). Then E1 protein can form a thiol-ester bond between a critical cysteine amino acid in E1 and the carboxyl-terminal glycine amino acid of ubiquitin. E1 is able to carry two ubiquitins, an ubiquitin adenylate and an ubiquitin linked by a thiol-ester bond to E1. E1 transfers the ubiquitin to an ubiquitin conjugating E2 enzyme, mediated through a cysteine in the active site of an E2, through transesterification. Then E2 either transfers the ubiquitin directly to the conserved, critical cysteine residue in the HECT domain of HECT family E3 ubiquitin ligases or forms an isopeptide bond between the glycine in ubiquitin and an internal lysine residue on the substrate protein or a growing ubiquitin chain on the substrate protein. This general process is illustrated in Figure 1.2. As E3 ubiquitin ligases provide

specificity in the ubiquitination process, more than 600 genes encode E3 ligases whereas about 40 genes are used to encode E2 enzymes (Deshaies and Joazeiro 2009). While there are some E3-E2 combinations that are restricted, most E3 ligases can interact with multiple E2 enzymes. Some combinations also include a multi-subunit complex of E3 ligases, such as the SCF (Skp, Cullin, F-box containing complex) or APC (Anaphase Promoting Complex), each of which can include several individual ubiquitin ligases in a complex.

Figure 1.2. Ubiquitination

Adapted from: (Fang and Weissman 2004) and (Eldridge and O'Brien 2010)



E3 Ubiquitin Ligases

E3 ubiquitin ligases complete the ubiquitination process by attaching either monomeric ubiquitin or a multi-ubiquitin chain to specific target proteins. There are several different domains of E3 ubiquitin ligases, which are unique to ubiquitin ligases of different families. These distinctive domains include HECT (Homologous to E6-AP Carboxyl Terminus), RING (Really Interesting New Gene), U-box, PHD (Plant Homeo-Domain), and LAP (Leukemia-Associated Protein) domains, which are all critical in mediating the transfer of ubiquitin to protein substrates. The different families of E3 ubiquitin ligases and their distinctive features are summarized in Figure 1.3. HECT domain-containing E3 ubiquitin ligases include a critical cysteine amino acid about 35 amino acids from the carboxy-terminus in their HECT domain of about 350 amino acids, which allows for ubiquitin to be transferred directly onto the E3 ligase before ubiquitination of the substrate occurs (Huibregtse, Scheffner et al. 1995). This also permits HECT E3 ligases to ubiquitinate themselves. The RING finger domain is designated by the presence of eight conserved cysteine and histidine amino acids which utilize two zinc ions to transfer ubiquitin onto a substrate. This domain is defined as Cys-X_2-Cys-$X_{(9-39)}$-Cys-$X_{(1-3)}$-His-$X_{(2-3)}$-Cys-X_2-Cys-$X_{(4-48)}$-Cys-X_2-Cys, in which X is defined as any amino acid (Borden and Freemont 1996). The PHD finger is similar to the RING finger motif, but has a histidine in the fourth position rather than a cysteine (Capili, Schultz et al. 2001).

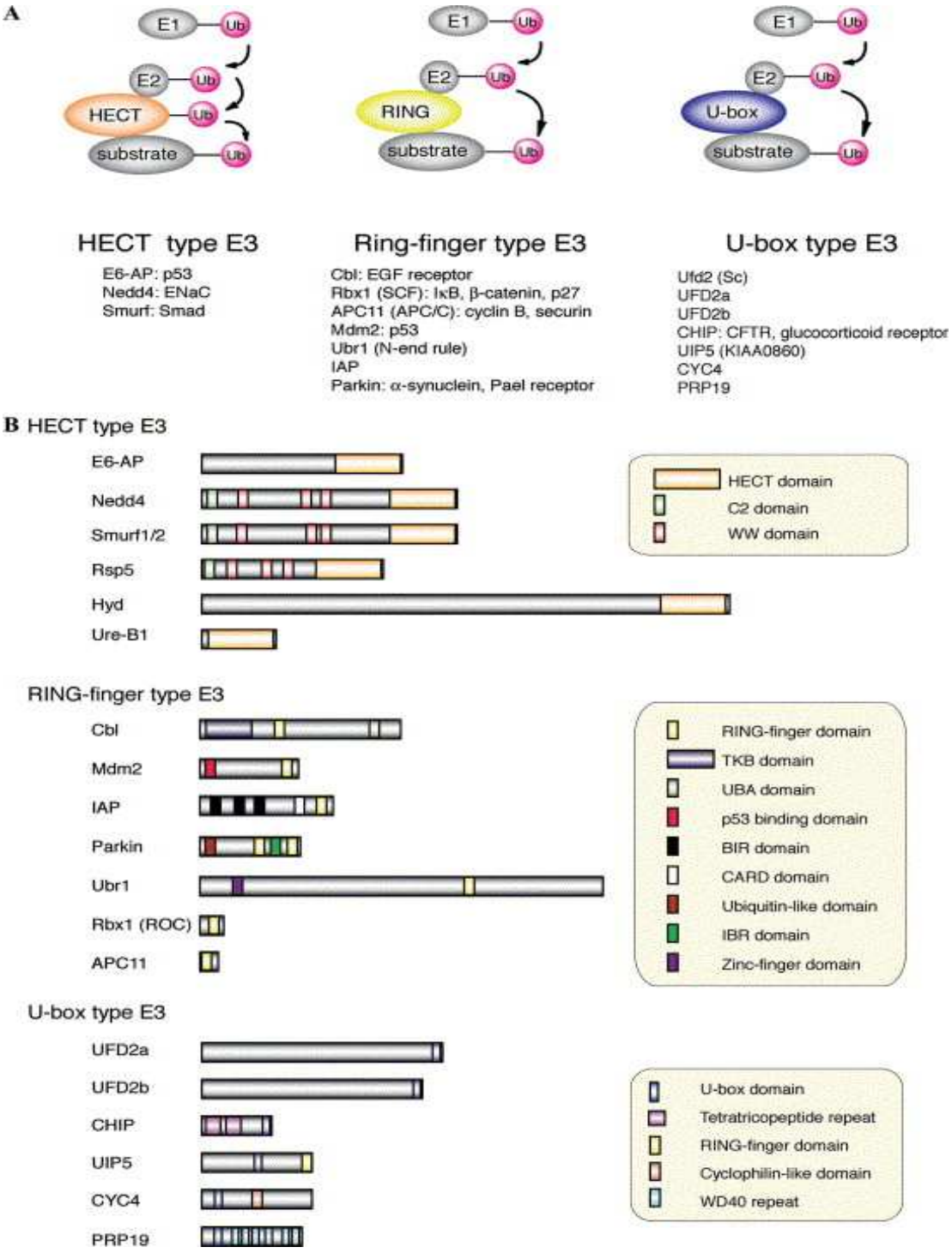
E3 ubiquitin ligases are notoriously large proteins with multiple domains allowing for protein-protein interactions, which permit the interaction with

potential ubiquitination targets. Unlike kinases, which have a catalytic binding pocket that is critical for the binding of ATP, an active site in a binding pocket is not present in E3 ubiquitin ligases. This makes the discovery of E3 ubiquitin ligase small molecule inhibitors extremely difficult. While targeting the protein/protein and protein/ubiquitin interaction domains may seem relevant, the promiscuity of ubiquitin ligases to interact with multiple E2 enzymes and substrates makes this a difficult proposition. Nutlin-3, a *cis*-imidazoline analog, binds to the E3 ubiquitin ligase Mdm2 to prevent its interaction with the tumor suppressor p53. The inhibition of this interaction leads to the accumulation of p53 to promote apoptosis (Vassilev, Vu et al. 2004). Clinical trials have not been completed on this inhibitor and the effects of this inhibitor on normal cells has yet to be determined (Secchiero, Bosco et al. 2011).

E3 enzymes are the ultimate determining factor for substrate specificity in the ubiquitination process (Hershko, Heller et al. 1986). Specificity is also conferred by the type of the ubiquitin attachment, including which lysine in the protein is targeted for ubiquitination, the type of isopeptide linkage (such as Lys 6, Lys 48, Lys 63, etc.), the addition of one ubiquitin protein, or the addition of a multi-ubiquitin chain.

Figure 1.3. E3 Ubiquitin Ligases

From (Hatakeyama and Nakayama 2003)



26S proteasome

The 26S proteasome consists of a 20S proteasome in its catalytic core and two 19S caps on either end of the catalytic core (Arrigo, Tanaka et al. 1988, Hoffman, Pratt et al. 1992). The 19S cap, also known as PA700, recognizes ubiquitinated proteins that have been targeted for degradation. Ubiquitin is removed and recycled before proteins enter the proteolytic core of proteasomes. The 20S proteasome utilizes ATP to degrade folded proteins, unfolded proteins, and peptides. In order to prevent random degradation of proteins in the cell, the catalytic core is insulated by the two 19S caps on either end of the channel formed by the proteasome core (Glickman, Rubin et al. 1998).

The 19S caps are made up of several different subunits to provide the complex with a range of activities including deubiquitination, recognition of ubiquitin, ATPase, and reverse chaperone activity to allow target proteins to be unfolded and funneled into the proteasome pore. The 19S cap is composed of nine subunits in its base complex and eight subunits in its lid complex (Fang and Weissman 2004). The 20S proteasome core is comprised of four stacked rings with seven subunits, each ring made of either α type or β subunits (αββα). The rings composed of β subunits are dependent on the formation of the α subunit ring first. The two inner-most rings are composed of β subunits, which possess catalytic activity. The β subunits have a critical threonine amino acid at the amino terminus of the protein, which is exposed after a pro-sequence is cleaved off the protein. The exposed threonine acts as a nucleophile to cleave peptides (Kisselev, Songyang et al. 2000). Different types of protease activity (trypsin-like,

chymotrypsin-like, and caspase-like) are dependent of different types of β subunits (Heinemeyer, Fischer et al. 1997, Jager, Groll et al. 1999). The proteasome will cleave proteins until the peptides that remain are small enough to diffuse out of the proteasome.

Most protease inhibitors are peptides which mimic the transition state of a peptide undergoing proteolysis, but in this case the peptide is unable to be cleaved. These inhibitors can either reversibly or irreversibly inhibit the proteasome, dependent on a covalent bond forming between the peptide inhibitor and the proteasome. For example, MG132 is a reversible proteasome inhibitor with the peptide sequence Z-Leu-Leu-Leu-CHO, which is a peptide aldehyde. Most proteasome inhibitors form a hemiacetyl complex with the critical threonine of the β subunits (Rock, Gramm et al. 1994).

Deubiquitinating Enzymes

Deubiquitinating enzymes include ubiquitin carboxy-terminal hydrolases (UBHs) and ubiquitin-specific processing proteases (UBPs). UBPs generally remove ubiquitin from proteins with a multi-ubiquitin chain. UBHs are generally associated with generating free monomeric ubiquitins, either from a multi-ubiquitin chain on targeted proteins or from ubiquitin genes that are translated into a poly-ubiquitin chain (Kim, Park et al. 2003). There are approximately 79 deubiquitinating enzymes, each with specificity (Nijman, Luna-Vargas et al. 2005). The removal of ubiquitins can prevent a protein from being degraded.

EDD

EDD was initially discovered in humans as a progestin-regulated HECT family E3 ubiquitin ligase that was identified by differential display in T47D breast cancer cells (Callaghan, Russell et al. 1998). Thus, EDD is an abbreviation for E3 ubiquitin ligase identified by Differential Display. Other names for EDD include *hyd* (hyperplastic discs gene) and *ubr5* (ubiquitin protein ligase E3 component N-recognin 5). The first ortholog of this gene was discovered in *Drosophila melanogaster* as the hyperplastic discs' tumor suppressor gene (Mansfield, Hersperger et al. 1994). In *Drosophila melanogaster*, EDD crucially regulates proliferation and differentiation via the hedgehog and decapentaplegic signaling pathways. EDD has also been identified in rat testis during postnatal development and was characterized as Rat100 (Oughtred, Bedard et al. 2002). In rat testis, mutants of the *edd* gene cause defects in spermatogenesis. EDD is ubiquitously expressed in humans with the highest levels present in testis, brain, pituitary, and kidney. Significant levels of EDD expression were also detected in the uterus, placenta, stomach, and prostate (Callaghan, Russell et al. 1998).

The *edd* gene is located on chromosome 8q22.3 (Callaghan, Russell et al. 1998). The EDD protein is over 300 kDa in size and encodes several functional domains which are depicted in Figure 1.4. EDD contains two nuclear localization signals, a Poly A Binding Protein homology domain, two regions for protein-protein interactions (UBA, UBR), several potential steroid receptor binding motifs (indicated by * in Figure 1.4), and a HECT domain with a conserved cysteine residue, which is critical for the transfer of ubiquitin onto EDD and subsequently

onto a target protein. The resolved structure of the HECT domain of EDD is depicted in Figure 1.5. The structure of the UBA domain of EDD is illustrated in Figure 1.6. EDD interacts with importin α5 to transport EDD into the nucleus (Henderson, Russell et al. 2002). This interaction occurs at EDD's nuclear localization sequences, one of which is simple and the other bipartite.

Figure 1.4. Domains of EDD from (Henderson, Russell et al. 2002)

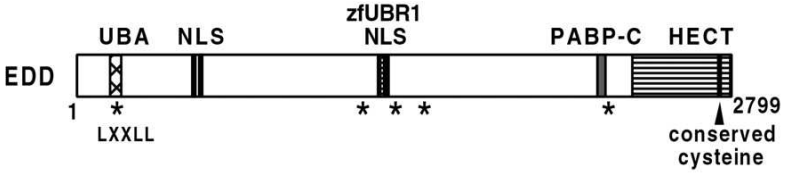


Figure 1.5. C-Terminal HECT Domain of EDD from (Matta-Camacho, Kozlov et al. 2012)

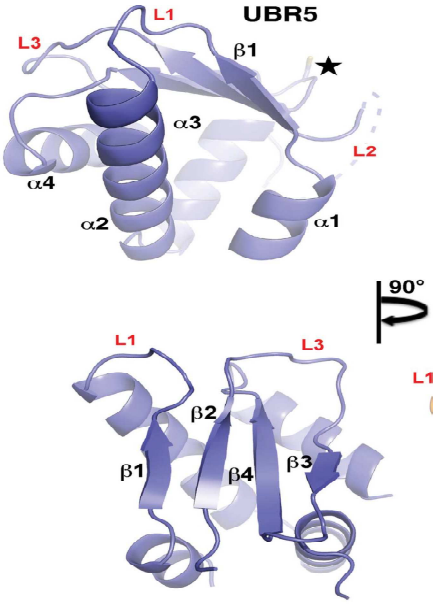
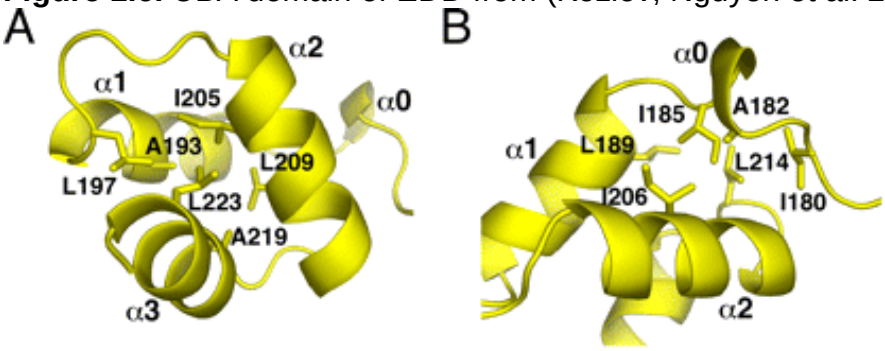
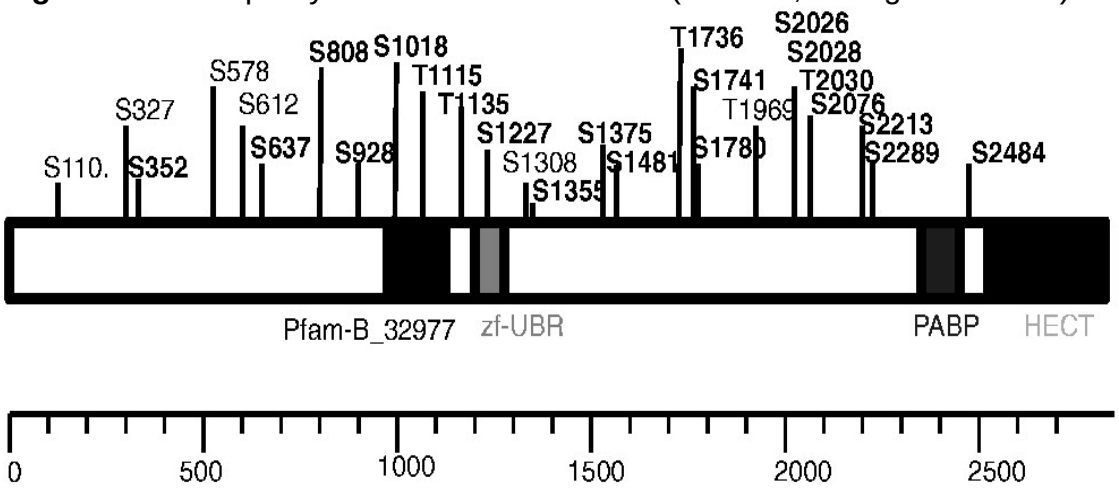


Figure 1.6. UBA domain of EDD from (Kozlov, Nguyen et al. 2007)



My mentor, Dr. Scott Eblen, discovered that EDD is a direct substrate of ERK2 (Eblen, Kumar et al. 2003). Our lab has determined several sites of phosphorylation on EDD, which is depicted in Figure 1.7. Kinases, other than ERK2, that phosphorylate EDD are still unknown at this time.

Figure 1.7. Phosphorylation sites of EDD from (Bethard, Zheng et al. 2011)



EDD has multiple functions in the cell, which can be dependent or independent on its ubiquitin ligase activity (Figure 1.9). One of EDD's ubiquitination targets is poly A binding protein interacting protein 2 (Paip2), an inhibitor of PABP activity. EDD targets Paip2 for degradation, which increases overall translation by increasing the activity of PABP (Yoshida, Yoshida et al. 2006). Another interaction which permits EDD to affect translation is an interaction with α4 phosphoprotein, which is a component of the mTOR (mammalian target of rapamycin) pathway, promoting translational initiation (McDonald, Sangster et al. 2010). This interaction occurs through the PABP-C domain of EDD. The α4 phosphoprotein can also interact with PABP itself.

β-catenin is the first protein that has been shown to be ubiquitinated by EDD, but not degraded as a consequence. EDD ubiquitinates β-catenin to increase its stability with ubiquitin chains linked by lysine residues 29 and 11 (Hay-Koren, Caspi et al. 2011). This allows for an increase in transcription of Wnt-regulated and β-catenin regulated genes. Many of these genes are linked with the progression of cancer and poor prognosis in cancer patients. However, EDD was identified as an interacting partner of adenomatous polyposis coli (APC), which allows for the stabilization of APC to enhance its protein expression (Ohshima, Ohta et al. 2007). This also promotes the inhibition of β-catenin, a downstream target of APC, which suggests that EDD may have a role as a tumor suppressor in colorectal cancer, where alterations in APC are prevalent.

Another ubiquitination target of EDD is DNA Topoisomerase II-binding protein (TopBP1). In cells without DNA damage, EDD mediates the ubiquitination and subsequent degradation of TopBP1 (Honda, Tojo et al. 2002). In response to DNA damage, TopBP1 is protected from ubiquitination due to phosphorylation to promote its co-localization with γ-H2AX at sites of DNA damage. TopBP1 is then able to protect the ends of the damaged DNA and facilitate their repair. Thus, EDD manages TopBP1 protein levels to coordinate DNA damage response.

EDD also interacts with several proteins to modulate their activity. Henderson et al. reported an interaction between EDD and CHK2, a DNA damage checkpoint kinase (Henderson, Munoz et al. 2006). This interaction is required for the phosphorylation of CHK2 on threonine 68 and the resulting activation of CHK2 as a result of DNA damage detection. The CHK2 kinase

phosphorylates proteins in cells with damaged DNA to promote mitotic arrest, DNA damage repair, and apoptosis if the damage is irreparable (Bartek, Falck et al. 2001, Falck, Mailand et al. 2001, Stevens, Smith et al. 2003). EDD is critical to this response. Knockdown of EDD in cells prevents DNA damage response and CHK2 activation. Similarly, the depletion of EDD in cells leads to defective DNA damage checkpoint activation resulting in mitotic catastrophe. Loss of EDD increased protein expression of E2F1 and Cdc25A/C, while decreasing expression of p27 and p21, with or without the presence of DNA damage (Munoz, Saunders et al. 2007). The disruption of these cell cycle checkpoints leads to premature mitosis in the presence of DNA damage, buildup of polyploid cells, and ensuing apoptosis. Other indications that EDD is involved in regulating DNA include its interaction with CIB1, a DNA-dependent kinase-interacting protein, and interaction with PMS1 and PMS2 during mismatch repair (Henderson, Russell et al. 2002, Cannavo, Gerrits et al. 2007). Furthermore, EDD ubiquitinates TopBP1 (topoisomerase IIβ-binding protein) as mentioned above.

In addition to EDD's role in the DNA damage pathway, EDD was recently shown to cooperate with TRIP12, another E3 ubiquitin ligase, to control accumulation of RNF168 (Gudjonsson, Altmeyer et al. 2012). RNF168 is a critical component of a complex to promote the ubiquitination of histones as a response mechanism to DNA damage. The presence of EDD and TRIP12 are crucial to maintain RNF168 levels to prevent extensive chromatin ubiquitination from spreading to undamaged chromosomes. Confirming the results of this study,

Okamoto et al. then demonstrated that inhibition of RNF168 by EDD is able to impede chromosome end-to-end fusions (Okamoto, Bartocci et al. 2013). This ultimately allows for a complex including TRF2 to protect chromosome ends. These studies confirm a role for EDD is maintaining chromosome integrity.

EDD interacts with a dual specificity tyrosine phosphorylation regulated kinase, DYRK2. DYRK2 acts as a scaffold for EDD, VPRBP, and DDB1 proteins in an E3 ubiquitin ligase complex (Maddika and Chen 2009). The interaction of these proteins, facilitated by DYRK2, mediates the phosphorylation and degradation of katanin p60. EDD is the catalytic E3 ubiquitin ligase to regulate the ubiquitination of katanin p60. Katanin p60 is a microtubule-severing agent, which is critical during anaphase of mitosis to allow for segregation of chromatids. If ubiquitination of katanin p60 is prevented, cells become polyploid. Additionally, through interaction with DYRK2, EDD promotes the ubiquitination and degradation of TERT, a catalytic subunit of telomerase (Jung, Wang et al. 2013). Phosphorylation of TERT by DYRK2 during the G2/M phase of the cell cycle prompts interaction and ubiquitination from EDD leading to TERT degradation. Since TERT is a subunit of telomerase, this causes inhibition of telomerase.

There have been conflicting reports regarding an interaction between EDD and the tumor suppressor protein p53. Research by Ling and Lin suggests that EDD prevents ATM (ataxia telangiectasia mutated) mediated phosphorylation of p53 on serine 15 (Ling and Lin 2011). The phosphorylation of p53 on this residue by ATM is required for activation of p53 regulated genes in response to DNA

damage. EDD silencing prompts p53 phosphorylation to activate p53 target genes even in the absence of DNA damage. This results in a decrease in cells entering S-phase. Previously, Munoz et al. demonstrated opposite results in their research indicating that depletion of EDD leads to an increase in the percentage of cells entering S-phase as well as opposite effects on p21 expression levels (Munoz, Saunders et al. 2007). Also, Saunders et al. showed that the phenotype in an EDD knockout mouse is not dependent on p53 expression, as shown in a p53 null mouse (Saunders, Hird et al. 2004). It was proposed that the differing conclusions from the two labs mentioned above are dependent on the cell types used in the experiments (Watts and Saunders 2011).

EDD interacts with Ago1, Ago2, GSPT1/2, ATXN2, and DDX6 proteins in the Argonaute-miRNA complex through its PABP-C domain (Su, Meng et al. 2011). Su et al. demonstrated that EDD is required for miRNA mediated silencing of genes through this interaction in mouse embryonic stem cells. This interaction is independent of ubiquitin ligase activity. MicroRNAs bind to partially complementary mRNAs to promote mRNA degradation and prevention of translation in targeted mRNAs.

Through mass spectroscopic analysis, EDD was identified as a part of the HPV-18 (human papillomavirus) E6/E6AP complex, which is responsible for ubiquitination of substrates to target them for degradation (Tomaic, Pim et al. 2011). Loss of EDD in this complex stimulates the activity of the complex to increase proteolysis while higher EDD levels provide protection for the targeted substrates. Tomaić et al. proposed that the alternations in EDD levels may occur

due to progression through the viral life cycle to allow for degradation of assorted protein substrates. This indicates a role for EDD in HPV driven malignancies. Another study indicating a role for EDD in the regulation of the progression of cancer found that the *edd* gene was upregulated after a second exposure of ultraviolet radiation in human keratinocytes (Gupta, Chakrobarty et al. 2006). The *edd* gene was subsequently downregulated in colony forming cells which were exposed to ultraviolet radiation multiple times. The mechanism behind this phenomenon was not elucidated.

As detailed above, EDD has many cellular functions dependent on protein interactions and ubiquitination of target proteins (Figure 1.8). On a physiological level, EDD is highly associated with vascularization and the function of the protein myocardin. *Edd* knockout mice embryos (embryonic day 8.5-10.5) demonstrated an inability to develop the yolk sac and allantoic vasculature (Saunders, Hird et al. 2004). The defective development of the extra-embryonic environment resulted in lack of proliferation and increased apoptosis. While the knockdown of *edd* results in embryonic lethality, heterozygous mice developed normally and were able to produce offspring. EDD was also identified as an interacting partner for the transcription factor myocardin (Hu, Wang et al. 2010). EDD cooperates with myocardin to promote its stabilization and to induce expression of genes to regulate the differentiation of smooth muscle cells. Recently, a chromatin binding profiling experiment identified that EDD regulates the expression of ACVRL1, a regulator of angiogenesis (Chen, Yang et al. 2013). ACVRL1 is a serine/threonine protein kinase that responds to the TGF-β ligand.

The transcriptional regulation at the ACVRL1 promoter by EDD is required for blood vessel development and motility of endothelial cells during angiogenesis. This regulation of transcription is independent of EDD's ubiquitin ligase activity.

Interaction with the progesterone receptor allows EDD to potentiate expression of progestin-induced genes (Henderson, Russell et al. 2002). EDD also serves as a coactivator for vitamin D receptor-mediated transcription. EDD's ability to act as a transcriptional coactivator for specific hormone receptors is independent of its ubiquitin ligase function. Since EDD is a progestin-regulated gene itself, this grants the opportunity for a positive feedback loop.

Figure 1.8. Cellular Functions of EDD

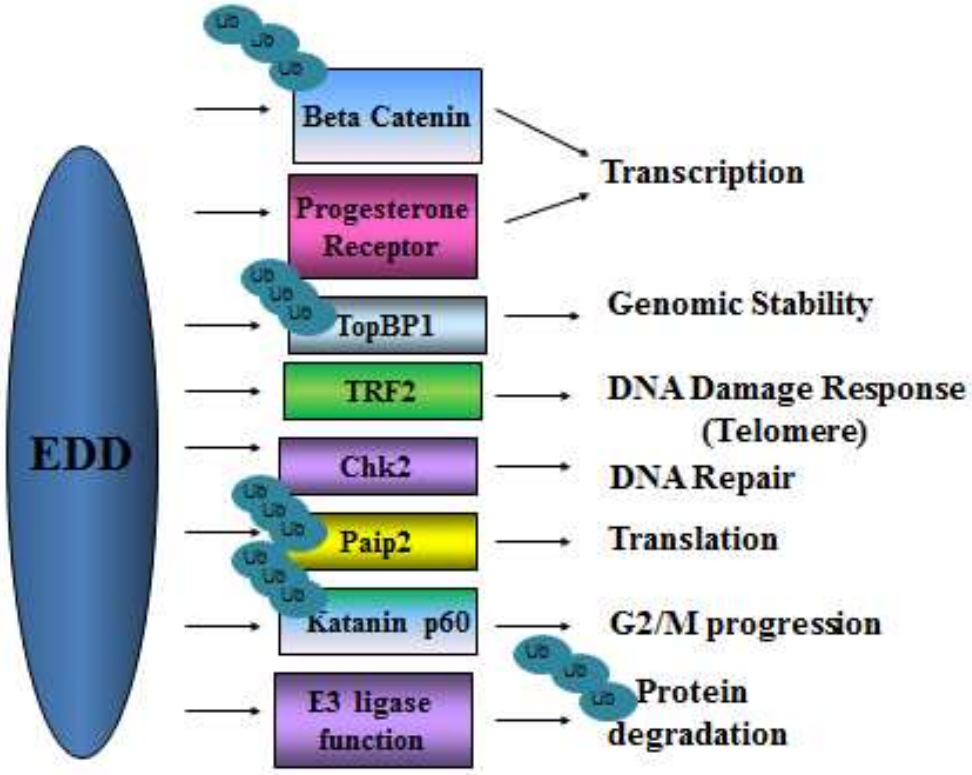
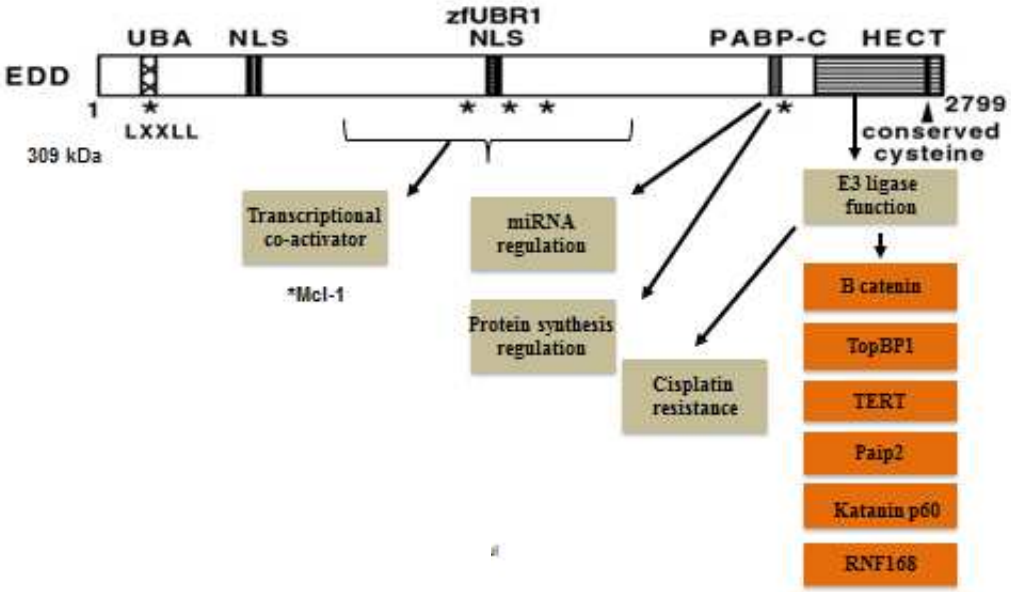


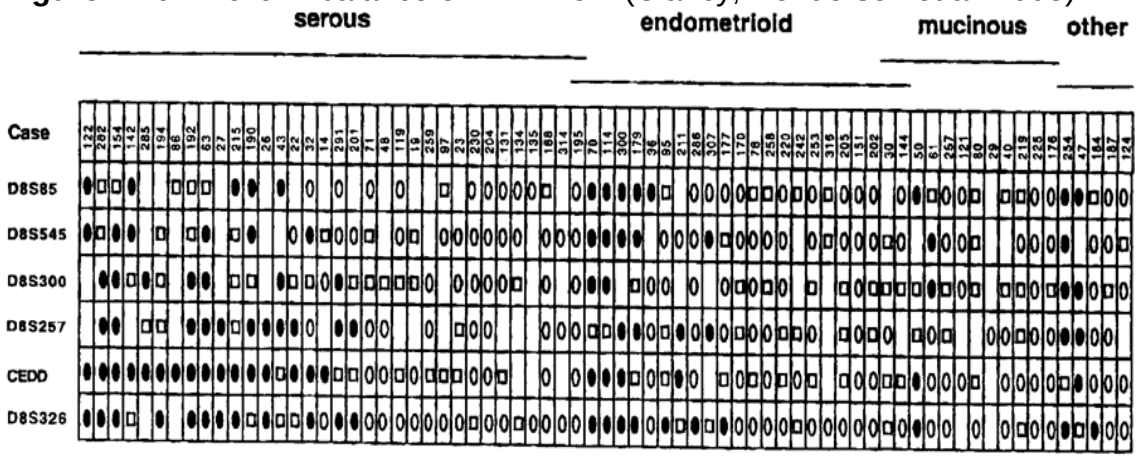
Figure 1.9. Separate Roles of EDD – Dependent or Independent of Ubiquitin Ligase Function (Modified from (Henderson, Russell et al. 2002))



EDD in Cancer

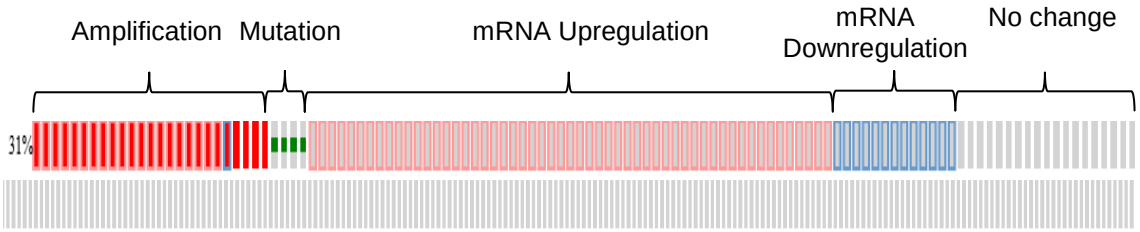
Truncation mutants of EDD are frequently found in gastric and colorectal cancers (Mori, Sato et al. 2002). The gene locus of *edd* is overamplified in several cancers including ovarian, breast, hepatocellular carcinoma, and metastatic melanoma (Fuja, Lin et al. 2004). Another study by Clancy et al. also demonstrated that EDD is overexpressed in ovarian and breast cancers (Clancy, Henderson et al. 2003). Clancy et al. analyzed several different cancers for allelic imbalance of EDD and found that 42% of all cancers in the study had either allelic gain or loss of the *edd* gene. While *edd* overexpression is rare in benign and borderline ovarian cancers, 47% of all types of this cancer overexpress *edd*, with 73% of serous ovarian cancers, the most prevalent form of ovarian cancer, overexpressing *edd*. This information is depicted in Figure 1.10. *Edd* is also overexpressed in oral squamous cell carcinoma (50%), hepatocellular carcinoma (46%), breast cancer (31%), and metastatic melanoma (18%). In ovarian cancer, EDD mRNA levels are often upregulated or downregulated as well, as demonstrated in Figure 1.11. Approximately 31% of 316 ovarian serous cystadenocarcinoma tumor samples have alterations at the level of mRNA (2011).

Figure 1.10. Allelic Imbalance of EDD from (Clancy, Henderson et al. 2003)



"Key: ●allelic imbalance, ○heterozygote, □uninformative homozygote, gap denotes no data available"

Figure 1.11. EDD levels are regulated at the mRNA level through upregulation or downregulation (Adapted from www.cbioportal.org/ Ovarian Serous Cystadenocarcinoma (TCGA, Nature) (2011))



Similarly, a gene cluster on chromosome 8q22, containing *edd* and *grhl2*, was identified to suppress death receptor expression (Dompe, Rivers et al. 2011). This research also revealed that EDD is overexpressed in several cancer cell lines that are resistant to apoptosis mediated by death-receptor activation. Specifically, EDD and GRHL2 reduced the expression of the death receptors Fas and DR5. Induction of apoptosis by death receptors such as Fas receptor and TRAIL receptor are detailed in the "Apoptosis" section, which describes the mechanism of extrinsic apoptosis (vide infra). Silencing of EDD, leading to increased expression of Fas and DR5, was shown to sensitize cancer cell lines

to ligand induced death receptor activation resulting in apoptosis. This research suggests that overexpression of EDD, and GRHL2, may offer tumors a mechanism to evade apoptosis.

EDD is implicated in the activation of necroptosis, a programmed form of necrosis which is caspase-independent. In response to caspase inhibition, RIP1 kinase and EDD were discovered to mediate JNK activation, resulting in the transcription of TNFα (Christofferson, Li et al. 2012). EDD interacts with RIP1 and potentially stabilizes this kinase. Through EDD's interaction with RIP1, JNK is activated through an unidentified mechanism, which promotes TNFα transcription. Transcription of TNFα is mediated most likely through the transcription factors AP-1 and SP1. EDD is required for the production of TNFα due to caspase inhibition, but EDD does not have a role in TNFα-mediated necroptosis. Regulation of TNFα may also have roles in apoptosis and inflammation, although this has not been addressed.

High expression of EDD in women with serous ovarian carcinoma, who showed an initial response to chemotherapy, is associated with a two-fold increased risk of recurrence and death (O'Brien, Davies et al. 2008). Even though protein expression of EDD did not correlate with cisplatin resistance, O'Brien et al. demonstrated in a cisplatin-resistant ovarian cancer cell line (A2780-cp70) in which siRNA-mediated knockdown of EDD increased sensitivity to cisplatin. High EDD expression in serous ovarian tumors reduced the median relapse time by 2.2 months in these patients (from 17.3 to 15.1 months in patients with low EDD tumor expression). The increased risk of relapse,

associated with high EDD expression, decreased median overall survival of these patients to 33.2 months as compared to 42.5 months for patients with low EDD expression (O'Brien, Davies et al. 2008). These results emphasize the significance of EDD in ovarian cancer as well as a potential role in mediating cisplatin resistance.

Apoptosis

Apoptosis is defined as a normal cellular process of programmed death. Characteristics of apoptosis include membrane blebbing, DNA fragmentation, condensation of the chromatin, and cellular shrinkage. Carl Vogt was the first to describe the principle of apoptosis in 1842, but apoptosis was not characterized until 1965 (Clarke and Clarke 2012). John Kerr, Alastair Currie, and Andrew Wyllie defined apoptosis using an electron microscope (Kerr, Wyllie et al. 1972). Within tissues, apoptotic cells divide themselves into smaller membrane bound bodies which undergo phagocytosis from neighboring cells or other phagocytic cells. This maintains tissue homeostasis. Apoptosis is a highly regulated process and is controlled by a genetically defined program. Research in *Caenorhabditis elegans* first identified the critical genes involved in mediating apoptosis (*nuc* 1, *ced* 3, *ced* 4, *ced* 9) (Horvitz 1999). These genes were later found to be homologous to the human anti-apoptotic and apoptotic proteins described later (vide infra).

The stimulus for apoptosis determines the genetic program that is activated in the cell to trigger apoptosis or, in some cases, overcome the cell's ability to prevent apoptosis. Extrinsic apoptosis is typically triggered through ligand binding and stimulation of a death receptor. Examples include tumor necrosis factor, Fas ligand, and tumor necrosis factor related apoptosis inducing ligand (TRAIL). These receptors have a cytoplasmic domain with a death domain which is critical to transmit the death signal from external stimuli to intracellular signaling pathways. Upon ligand binding, the death domain of the receptor can

recruit and bind to an adaptor protein. The adaptor protein (such as Fas Associated Death Domain and TRAIL Associated Death Domain) associates with procaspase 8 to form a death-inducing signaling complex (DISC) (Ashkenazi and Dixit 1998). This results in caspase 8 cleavage and activation. Caspases have proteolytic activity and are crucial for the cleavage and activation of the caspase cascade to execute apoptosis.

Intrinsic apoptosis is stimulated by stress, DNA damage, or withdrawal of growth factors, cytokines, or hormones. This pathway converges on the mitochondria where apoptosis is induced once a mitochondrial permeability transition pore is opened to decrease the mitochondrial membrane potential and release factors into the cytosol. The opening of this pore is tightly controlled by a balance of anti-apoptotic and pro-apoptotic Bcl-2 family proteins localized at the mitochondrial membrane. Anti-apoptotic proteins include Bcl-2, Bcl-XL, Bcl-XS, and Mcl-1. Pro-apoptotic proteins include Bax, Bak, Bad, Bim, Bid, Puma, Noxa, and Bik. The anti-apoptotic proteins exert their effect by heterodimerizing with the pro-apoptotic proteins. If the pro-apoptotic proteins are allowed to oligimerize, the mitochondrial pore will be formed from this oligomer. Once the mitochondrial pore is opened, factors such as cytochrome c, Smac/DIABLO, and endonucleases are released. Cytochrome c interacts with Apaf-1 and procaspase 9 to form an apoptosome resulting in caspase 9 cleavage and activation (Hill, Adrain et al. 2004).

The extrinsic apoptotic pathway can also intersect with the intrinsic pathway through caspase 8 mediated cleavage of the pro-apoptotic protein Bid to

tBid to allow for the formation of the mitochondrial pore (Li, Zhu et al. 1998). In the end, both the extrinsic and intrinsic pathways both converge on an execution pathway mediated through caspase 3, caspase 6, and caspase 7. These caspases cleave cellular substrates to induce apoptosis such as PARP, cytokeratins, endonucleases, and cytoskeletal proteins. This results in accumulation of DNA damage due to PARP cleavage, membrane blebbing, and cell shrinking due to degradation of cytoskeletal proteins and cytokeratins. Also, endonucleases degrade the chromosomal DNA. Ultimately, all of the normal cellular processes fail and apoptotic cells neglect maintenance of the phospholipid bilayer. This allows for phosphatidylserine to be exposed on the surface of these cells and their cellular fragments to serve as a signal for phagocytic uptake (Fadok, de Cathelineau et al. 2001).

Hypothesis

EDD regulates survival and cisplatin resistance in ovarian cancer cells and oral squamous cell carcinoma

Specific Aims

This hypothesis will be tested through the following specific aims:

B.3. Specific Aims

1. Identify the importance of EDD in regulating survival in ovarian cancer

1a: Establish if EDD knockdown with siRNA induces apoptosis in ovarian cancer cell lines

1b. Examine the changes in apoptotic and anti-apoptotic proteins due to EDD knockdown resulting in apoptosis

1c. Validate the importance of specific proteins by knockdown and overexpression to induce apoptosis in ovarian cancer cells

1d. Determine the mechanism by which EDD regulates alterations in specific apoptotic and/or anti-apoptotic proteins

2. Determine if EDD regulates cisplatin sensitivity in ovarian cancer

2a: Determine if EDD knockdown with siRNA and shRNA promotes cisplatin sensitivity

2b: Determine if EDD overexpression is sufficient to promote cisplatin resistance

2c: Establish if EDD is a therapeutic target in epithelial ovarian cancer through EDD knockdown experiments *in vivo*

3. Characterize the role of EDD in the regulation of cisplatin resistance in oral squamous cell carcinoma

3a. Generate inducible shRNA EDD knockdown cell lines

3b. Determine if EDD stable knockdown sensitizes squamous cell carcinoma cell lines to cisplatin treatment

Chapter 2

EDD regulates cellular survival in ovarian cancer cells

***Note: This chapter contains a portion of the paper:**
***Carcinogenesis.* 2014 May 1; volume 35, number 5, pages 1100-1109.**

EDD enhances cell survival and cisplatin resistance and is a therapeutic target for epithelial ovarian cancer.

Bradley A, Zheng H, Ziebarth A, Sakati W, Branham-O'Connor M, Blumer JB, Liu Y, Kistner-Griffin E, Rodriguez-Aguayo C, Lopez-Berestein G, Sood AK, Landen CN Jr, Eblen ST.

Introduction

Initial therapy for ovarian cancer involves surgical debulking combined with chemotherapy, which consists of platinum and paclitaxel; however, resistance to chemotherapy often occurs in recurrent tumors. One indicator of poor prognosis in recurrent ovarian cancer is the E3 ubiquitin ligase EDD (E3 ligase identified by differential display), a 300kDa nuclear phosphoprotein that we previously identified as a direct substrate of the MAP kinase extracellular signal-regulated kinase 2 (Henderson, Russell et al. 2002, Eblen, Kumar et al. 2003, O'Brien, Davies et al. 2008, Bethard, Zheng et al. 2011). EDD helps regulate the DNA damage response, mediates Chk2 kinase activation, and has been implicated in the S phase and G_2/M DNA damage checkpoints (Henderson, Russell et al. 2002, Henderson, Munoz et al. 2006, Munoz, Saunders et al. 2007, Gudjonsson, Altmeyer et al. 2012). EDD also acts as a transcriptional co-activator for the progesterone and vitamin D receptors, dependent upon its middle domain and independent of its E3 ligase activity (Henderson, Russell et al. 2002).

EDD protein is overexpressed or mutated in several solid tumors including ovarian, breast, hepatocellular, tongue, gastric, and melanoma (Mori, Sato et al. 2002, Clancy, Henderson et al. 2003, Fuja, Lin et al. 2004). EDD protein levels are low in benign ovarian tissue and borderline tumors, but overexpression is observed in 47% of ovarian cancer tumors overall, 73% of serous ovarian

tumors, and was associated with a 2-fold increased risk of recurrence and death in patients who had a favorable response to initial chemotherapy (Clancy, Henderson et al. 2003, O'Brien, Davies et al. 2008). In these results, I demonstrate that EDD directly contributes to cellular survival through upregulation of the anti-apoptotic protein myeloid cell leukemia sequence 1 (Mcl-1) in ovarian cancer cells.

Materials and Methods

Cell lines and antibodies

ES-2 and TOV21G cells were from Runzhao Li, OVCAR3 cells were from Kristen Atkins, A2780 cells were from Andrew Godwin, A2780ip2 cells were from Charles Landen, OVCAR5 cells were from Thomas Hamilton and IOSE cells were from Nelly Auersperg. COS-1, HeLa, and SKOV-3 cells were from American Type Culture Collection (ATCC, Manassas, VA). Mcl-1 stable cells were generated by transduction with pBabe or pBabe-Flag-Mcl-1 (Addgene) and puromycin-resistant clones (A2780ip2) or populations (ES-2) were selected. Antibodies [poly(ADP-ribose) polymerase (PARP), Bcl2 family proteins, actin] were from Cell Signaling (Danvers, MA) and the EDD (M19) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Lipofectamine 2000 (Invitrogen) was used for transfections, according to the manufacturer's protocol.

siRNA transfection

Cell lines were transfected with 45 nmol of control or EDD siRNA (Sigma–Aldrich). siRNA1: SASI_Hs01_00175227 (5′CCAUUUACCCUGGCUAGUA); siRNA2: SASI_Hs02_00348492 (5′GCGACUCUCCAUGGUUUCU). Mcl-1 siRNA: SASI_Hs01_00162656 (5′GUAAUAGAACUAUGACUGU). Bcl-xL siRNA: SASI_Hs01_00165963 (5′CUGAUUGGUGCAACCCUUA). Glycogen synthase kinase 3 beta (GSK-3β) siRNA1: SASI_ Hs01_00192106 (5′GGACUAUGU UCCGGAAACA) and GSK-3β siRNA2: SASI_Hs01_00192105 (5′CACUCAA GAACUGUCAAGU). Twenty nanomoles of Mcl-1, Bcl-xL and GSK-3β siRNA were used. Control siRNA was Universal Negative Control #1 (Sigma–Aldrich).

Western blotting

Floating and adherent cells were lysed with M2 lysis buffer containing 0.5% sodium dodecyl sulfate (Eblen, Catling et al. 2001). Typically, 65 µg of protein lysate was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (7–12% gradient gel) and immunoblotted proteins were visualized using enhanced chemiluminescence (Pierce). GSK-3β inhibitors used were LiCl (20mM, Sigma–Aldrich), TDZD-8 (10 µM) and L803-mts (20 µM, EMD Chemicals, Gibbstown, NJ). Cycloheximide (Sigma–Aldrich) was used at 50 µg/ml. For caspase inhibition, cells were co-treated with siRNA and either 25 µM pan caspase inhibitor Q-VD-OPH (R&D Systems, Minneapolis, MN) or the negative control Z-FA-FMK (BD Pharmingen, Franklin Lakes, NJ).

Crystal violet staining

Cells were fixed with 4% paraformaldehyde, stained with 0.05% crystal violet in 2% ethanol for 15 min, washed five times with phosphate-buffered saline and dried. Stained cells were solubilized with 2% sodium dodecyl sulfate in phosphate-buffered saline and absorbance was measured at 550 nm.

Quantitative real-time PCR

RNA was extracted using the Qiagen (Valencia, CA) RNeasy Plus Mini Kit and cDNA was synthesized using the Bio-Rad (Hercules, CA) iScript™ Advanced cDNA Synthesis Kit for RT-qPCR. Bio-Rad's SsoAdvanced™ SYBR® Green Supermix was used for quantitative real-time PCR on an Eppendorf (Hauppage, NY) Mastercycler Realplex 2. The average fold change of the test sample over control sample was determined for each experimental condition with

normalization to two housekeeping genes, actin and glyceraldehyde 3-phosphate dehydrogenase. The Mcl-1 primer was from Integrated DNA Technologies (Coralville, IA) (forward: 5′-AAAGAGGCTGGGATGGGTTT-3′, reverse: 5′-CAAAA GCAAGCAGCACATTC-3′). The actin primer used was from Real-Time Primers (forward: 5′-GGACTTCGAGCAAGAGATGG-3′, reverse: 5′-AGCACTGTGT TGGCGTACAG-3′) along with glyceraldehyde 3-phosphate dehydrogenase (forward: 5′-GAGTCAACGGATTTGGTCGT-3′, reverse: 5′-TTGATTTTGG AGGGATCTCG-3′).

Flow cytometry

Floating and adherent cells were fixed in ethanol and stained with propidium iodide (Molecular Probes, Eugene, OR). DNA content was determined by flow cytometry and sub-2n cells were counted as apoptotic. The Student's *t*-test was performed on three independent experiments done in duplicate.

Luciferase assays

HeLa cells were transfected with 40 ng TK *Renilla* luciferase, 400ng of the firefly luciferase plasmids p(−2389/+10)mcl-luc, p(−1289/+10)mcl-luc, p(−567/+10)mcl-luc, p(-70/+10)mcl-luc, or empty mcl-luc (Chao, Wang et al. 1998) and 2 μg of either wild-type or mutant Flag-EDD or empty vector. Luciferase assays were performed at 48hr using the Dual Luciferase Reporter Assay (Promega) on a Monolight 2010 Luminometer (Analytical Luminescence, Ann Arbor, MI). Firefly luciferase activity was normalized to *Renilla* luciferase. The results are a combination of four independent experiments done in triplicate. After averaging over experimental replicates, a two-sample *t*-test was conducted for each

luciferase plasmid testing the effect of EDD or EDD mutant versus vector. Cell lysates were immunoblotted for EDD and actin.

Results

EDD knockdown induces apoptosis in ovarian cancer cells

Immunoblotting lysates from ovarian cell lines showed high EDD expression in five of seven ovarian cancer cell lines compared with the preneoplastic IOSE398 cell line, with the highest expression in ES-2, OVCAR5 and A2780 cells (Figure 2.1A). To determine the effect of EDD knockdown, we transfected A2780ip2 (Figure 2.2A), ES-2 (Figure 2.2B) and OVCAR5 (Figure 2.2C) cells with control siRNA or one of two EDD siRNAs. EDD siRNAs knocked down EDD protein expression, with siRNA1 having the strongest effect. Interestingly, cells transfected with EDD siRNA showed a significant reduction in cell number in all three cell lines within 48hr, as measured by quantitation of crystal violet staining, with the exception of siRNA2 in ES-2 cells (Figure 2.2D). Loss of cell viability after EDD siRNA1 transfection increased from 24 to 72hr (Figure 2.2E). To determine whether EDD knockdown induced apoptosis, lysates from floating and adherent siRNA-transfected cells were immunoblotted for cleavage of PARP, a substrate of caspases and an indicator of apoptosis. Enhancement of cleaved PARP relative to total PARP (cleaved plus uncleaved) was observed in all three cell lines after EDD siRNA transfection (Figure 2.1B–D), with siRNA1 having a greater effect, coinciding with greater EDD knockdown, especially in ES-2 cells. A2780ip2 cells showed enhanced apoptotic sensitivity to EDD knockdown at earlier time points (Figure 2.1B). In addition, propidium iodide staining followed by flow cytometry showed significant apoptosis, measured by <2n DNA content, after 48hr of EDD knockdown in A2780ip2 (control = 5.8%;

EDD = 44.6%), ES-2 (control = 5.8%; EDD = 42.6%) and OVCAR5 (control = 5.9%; EDD = 22.6%) cells (Figure 2.1E). The induction of apoptosis showed a temporal increase in both A2780ip2 (Figure 2.1F) and ES-2 cells (Figure 2.1G). The relatively rapid induction of apoptosis suggested a short EDD half-life and strong requirement for cell survival. Cycloheximide experiments demonstrated the half-life of EDD protein was ~4hr in A2780ip2 cells (Figure 2.1H).

Figure 2.1

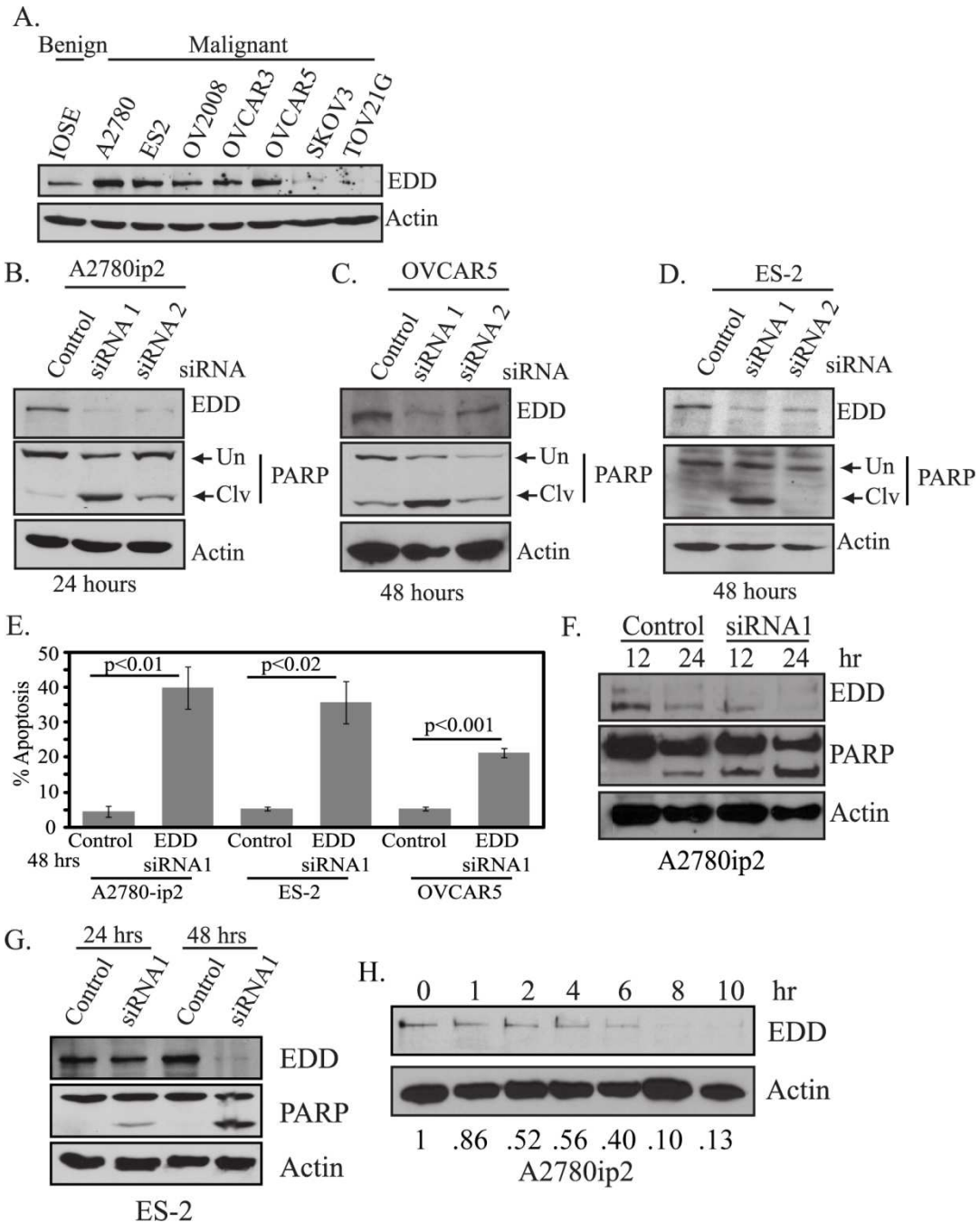

A.
Benign
Malignant
IOSE
A2780
ES2
OV2008
OVCAR3
OVCAR5
SKOV3
TOV21G
EDD
Actin
B.
A2780ip2
Control
siRNA 1
siRNA 2
siRNA
EDD
Un
Clv
PARP
Actin
24 hours
C.
OVCAR5
48 hours
D.
ES-2
48 hours
E.
% Apoptosis
p<0.01
p<0.02
p<0.001
48 hrs
EDD siRNA1
A2780-ip2
ES-2
OVCAR5
F.
Control
siRNA1
12
24
hr
A2780ip2
G.
24 hrs
48 hrs
ES-2
H.
0
1
2
4
6
8
10
1 .86 .52 .56 .40 .10 .13
A2780ip2

Figure 2.1. EDD is overexpressed in ovarian cancer cell lines and EDD knockdown induces apoptosis. (A) EDD expression was determined by immunoblotting lysates from ovarian cell lines. (B) A2780ip2, (C) OVCAR5 and (D) ES-2 cells were transfected with control siRNA or one of two siRNAs to EDD. After transfection for the indicated time, floating and adherent cells were harvested and cell lysates were immunoblotted for EDD expression and PARP. Uncleaved (Un) and cleaved (Clv) PARP are indicated with arrows. (E) Cells were transfected with control siRNA or EDD siRNA1 for 48hr and floating and adherent cells were stained with propidium iodide. Flow cytometry was used to determine the percentage of cells with sub-2n DNA content, an indicator of apoptosis. The results are from three independent experiments. (F) A2780ip2 cells were transfected with control siRNA or EDD siRNA1 for 12 or 24hr and lysates from floating and adherent cells were immunoblotted for EDD expression and PARP cleavage. (G) ES-2 cells were transfected with control siRNA or EDD siRNA1 for 24 or 48hr and lysates from floating and adherent cells were immunoblotted for EDD expression and PARP cleavage. (H) A2780ip2 cells were treated with 50 μg/ml of cycloheximide for the indicated time. Cell lysates were immunoblotted for EDD and actin. The number under each lane indicates the relative intensity of the EDD band compared with actin, with the amount in time zero set at 1.

Figure 2.2

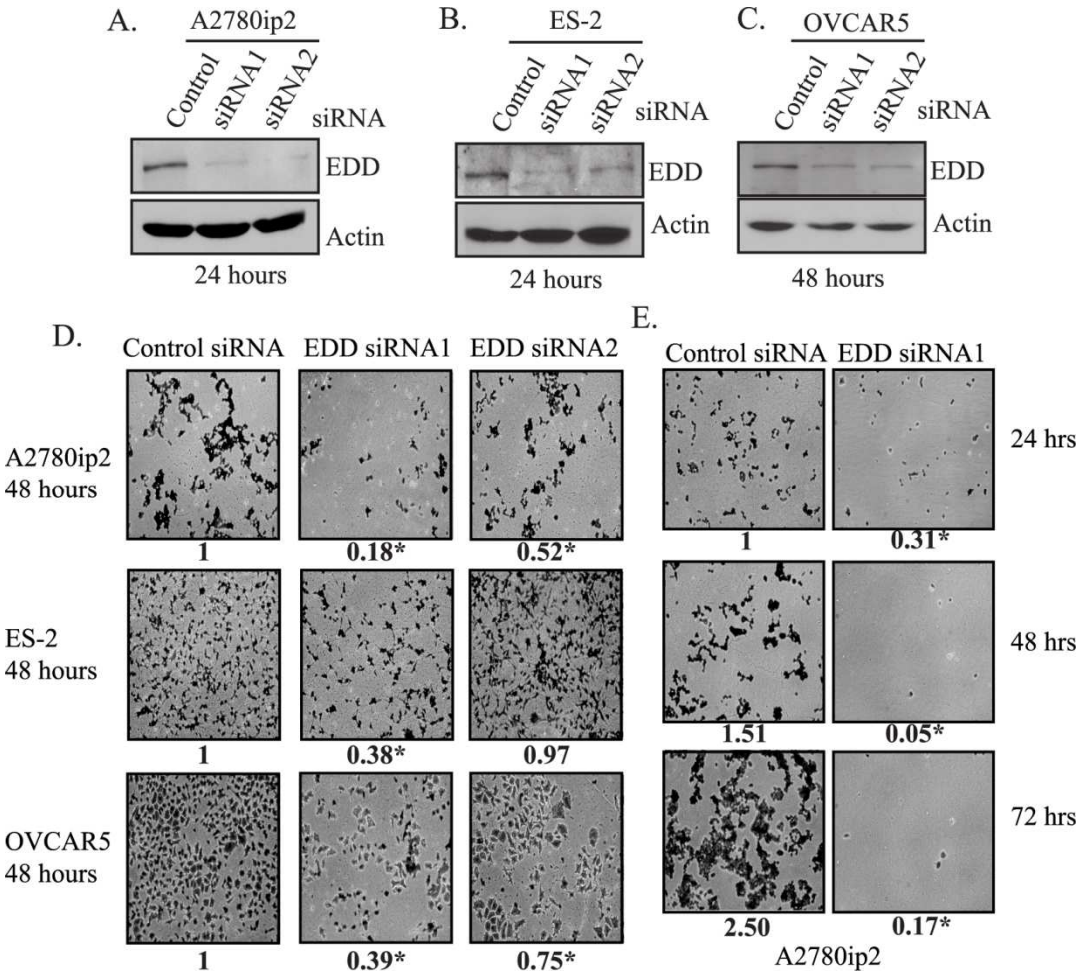

A.
A2780ip2
Control
siRNA1
siRNA2
siRNA
EDD
Actin
24 hours
B.
ES-2
Control
siRNA1
siRNA2
siRNA
EDD
Actin
24 hours
C.
OVCAR5
Control
siRNA1
siRNA2
siRNA
EDD
Actin
48 hours
D.
Control siRNA
EDD siRNA1
EDD siRNA2
A2780ip2
48 hours
1
0.18*
0.52*
ES-2
48 hours
1
0.38*
0.97
OVCAR5
48 hours
1
0.39*
0.75*
E.
Control siRNA
EDD siRNA1
24 hrs
1
0.31*
48 hrs
1.51
0.05*
72 hrs
2.50
0.17*
A2780ip2

Figure 2.2. EDD knockdown reduces cellular survival. (A) A2780ip2, (B) ES-2 and (C) OVCAR5 cells were transfected with control or EDD siRNAs for 24hr and immunoblotted for EDD expression. (D) EDD knockdown reduces cell viability. Cells were transfected with control or EDD siRNA for 24hr, fixed, stained with crystal violet, and photographed at 10X magnification. The cells were solubilized and crystal violet absorbance measured. The number under each photograph corresponds to absorbance relative to control siRNA in that cell line and statistical significance from control transfected cells ($p<0.05$) is indicated with an asterisk (*). The results are from three independent experiments. (E) A2780ip2 cells were transfected with control siRNA or EDD siRNA1 for 24, 48, or 72hr and processed as in (D).

EDD knockdown causes loss of Mcl-1 through a degradation- independent mechanism

To identify a potential mechanism of apoptosis induction, we immunoblotted siRNA-transfected cell lysates with antibodies to Bcl2 family members, which have both prosurvival and proapoptotic functions (Burlacu 2003, Ola, Nawaz et al. 2011). EDD knockdown resulted in specific downregulation of the prosurvival protein Mcl-1 in all three cell lines, correlating with increased PARP cleavage (Figure 2.3A), and Mcl-1 loss was detected using either EDD siRNA1 or siRNA2 (Figure 2.4A). Pretreatment of the cells with the pan caspase inhibitor Q-VD inhibited PARP cleavage and the loss of the proapoptotic caspase 3 substrate p53 upregulated modulator of apoptosis (Puma) upon EDD knockdown, but did not inhibit loss of Mcl-1, suggesting that Mcl-1 loss was not a consequence of caspase action or apoptosis induction (Figure 2.3B) (Hadji, Clybouw et al. 2010). To compare the requirements for EDD and Mcl-1 in cell survival, we transfected cells with siRNA against EDD, Mcl-1, the prosurvival protein Bcl-xL, or control siRNA. Apoptotic cells were identified by propidium iodide staining. EDD or Mcl-1 siRNA induced equal and significant induction of apoptosis in A2780ip2 (control = 7.6%; EDD siRNA1 = 42%; Mcl-1 = 41.4%; Bcl-xL = 16.9%) and ES-2 cells (control = 4%; EDD siRNA1 = 25.8%; Mcl-1 = 22.6%; Bcl-xL = 6.1%), whereas Bcl-xL knockdown induced less apoptosis that was only significantly different from control in A2780ip2 cells and much less than that induced by EDD or Mcl-1 siRNA (Figure 2.3C and D). Immunoblotting demonstrated knockdown of the targeted proteins and levels of PARP cleavage

that corresponded to the relative level of apoptosis observed by propidium iodide staining (Figure 2.3E). These data show that these ovarian cancer cell lines have the same survival requirement for EDD and Mcl-1.

To determine if EDD regulated survival by promoting Mcl-1 levels, we generated stable cell lines expressing either Flag-Mcl-1 or empty vector. Stable ES-2 populations (Figure 2.3F) and A2780ip2 clones with varying levels of Flag-Mcl-1 (Figure 2.3G) were selected with puromycin. Flag-Mcl-1 migrated slower than endogenous Mcl-1 on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Flag-Mcl-1 overexpression inhibited PARP cleavage upon EDD knockdown in both ES-2 (Figure 2.3H) and A2780ip2 (Figure 2.3I) stable lines compared with the vector control lines, with a dose-dependent effect of exogenous Mcl-1 expression on inhibition of PARP cleavage in the A2780ip2 clones. Interestingly, EDD knockdown induced loss of endogenous Mcl-1, but not expression of the exogenous Flag-Mcl-1 expressed from a cytomegalovirus promoter. Collectively, these results show that Mcl-1 overexpression protects cells from apoptosis upon EDD knockdown.

Mcl-1 protein stability is controlled in part through phosphorylation by GSK-3β, stimulating Mcl-1 ubiquitination by β-transducin repeat-containing protein, followed by proteosomal degradation (Ding, He et al. 2007). EDD binds to GSK-3β and stimulates its nuclear accumulation (Hay-Koren, Caspi et al. 2011). To determine if EDD binding to GSK-3β ‘protects’ Mcl-1 from GSK-3β-induced degradation, which would be lost upon EDD knockdown, we transfected parental A2780ip2 cells with EDD siRNA1 and treated the cells with the GSK-3β

inhibitors TZDZ, lithium chloride or L803-mts (Klein and Melton 1996, Phiel and Klein 2001, Martinez, Alonso et al. 2002, Kaidanovich-Beilin and Eldar-Finkelman 2006, Rao, Hao et al. 2007). GSK-3β inhibitors did not inhibit Mcl-1 downregulation or PARP cleavage upon EDD knockdown (Figure 2.4B). Furthermore, GSK-3β knockdown for 24hr prior to EDD knockdown with siRNA1 did not prevent the loss of Mcl-1 protein or inhibit PARP cleavage (Figure 2.4C), suggesting that Mcl-1 downregulation after EDD knockdown is GSK-3β independent.

Figure 2.3

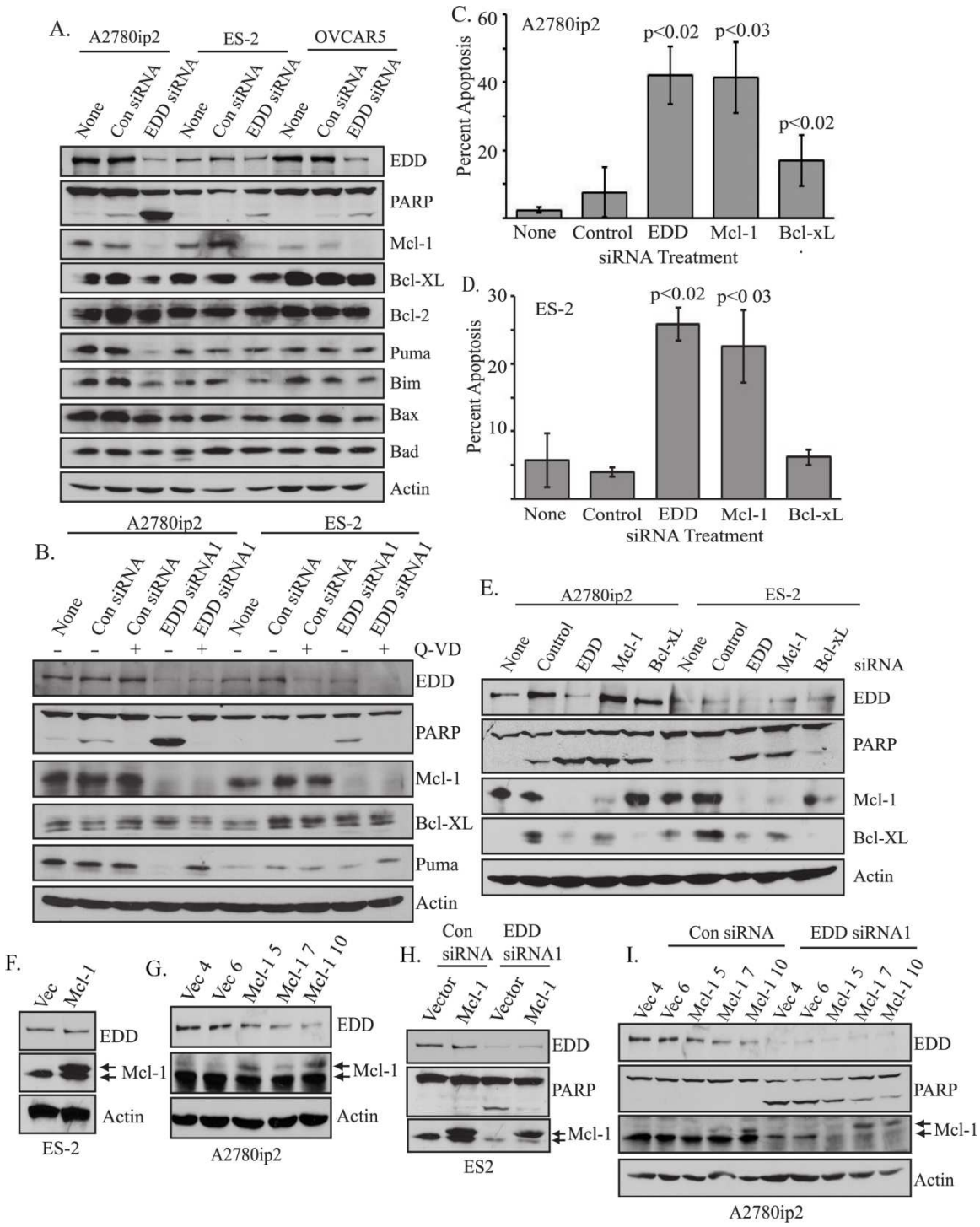

A.
A2780ip2
ES-2
OVCAR5
None
Con siRNA
EDD siRNA
EDD
PARP
Mcl-1
Bcl-XL
Bcl-2
Puma
Bim
Bax
Bad
Actin
B.
A2780ip2
ES-2
Con siRNA
EDD siRNA1
Q-VD
EDD
PARP
Mcl-1
Bcl-XL
Puma
Actin
C.
A2780ip2
Percent Apoptosis
p<0.02
p<0.03
p<0.02
None
Control
EDD
Mcl-1
Bcl-xL
siRNA Treatment
D.
ES-2
Percent Apoptosis
p<0.02
p<0 03
siRNA Treatment
E.
A2780ip2
ES-2
siRNA
F.
Vec
Mcl-1
ES-2
G.
Vec 4
Vec 6
Mcl-1 5
Mcl-1 7
Mcl-1 10
A2780ip2
H.
Con siRNA
EDD siRNA1
Vector
ES2
I.
A2780ip2

Figure 2.3. EDD downregulation decreases Mcl-1 protein levels, whereas Mcl-1 overexpression inhibits apoptosis upon EDD knockdown. (A) Cells were either untreated (none) or transfected with control or EDD siRNA1 for 24hr. Lysates from floating and adherent cells were immunoblotted for EDD, PARP and Bcl2 family members as indicated. (B) A2780ip2 and ES-2 cells were untreated (none) or transfected with control siRNA or EDD siRNA1 and simultaneously treated with either Q-VD-OPH pan caspase inhibitor (+) or the negative control Z-FA-FMK (−). After 24hr, floating and adherent cells were collected, lysed, run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotted for EDD, PARP, Mcl-1, Bcl-xL, p53 upregulated modulator of apoptosis, and actin. (C) A2780ip2 and (D) ES-2 cells were either untreated (none) or were transfected with the control siRNA, EDD siRNA1, or siRNA to Mcl-1 or Bcl-xL for 24hr. Floating and adherent cells were fixed, stained with propidium iodide, and the percentage of sub-2n cells determined by flow cytometry. *P* values represent significance compared with the control siRNA-transfected cells. (E) Cells were transfected with siRNA as in (D) for 24hr. Lysates from floating and adherent cells were immunoblotted to confirm knockdown and to determine PARP cleavage. (F) Stable populations of ES-2 cells and (G) stable clones of A2780ip2 cells expressing either pBabe vector (Vec) or pBabe-Flag-Mcl-1 (Mcl-1) were generated by retroviral transduction. Cell lysates were immunoblotted as indicated. Arrows indicate endogenous Mcl-1 and the slower-migrating Flag-Mcl-1. (H) Stable ES-2 or (I) A2780ip2 cells were transfected with control or EDD

siRNA1 for 24hr and cell lysates immunoblotted as indicated. Arrows indicate endogenous Mcl-1 and Flag-Mcl-1.

Figure 2.4

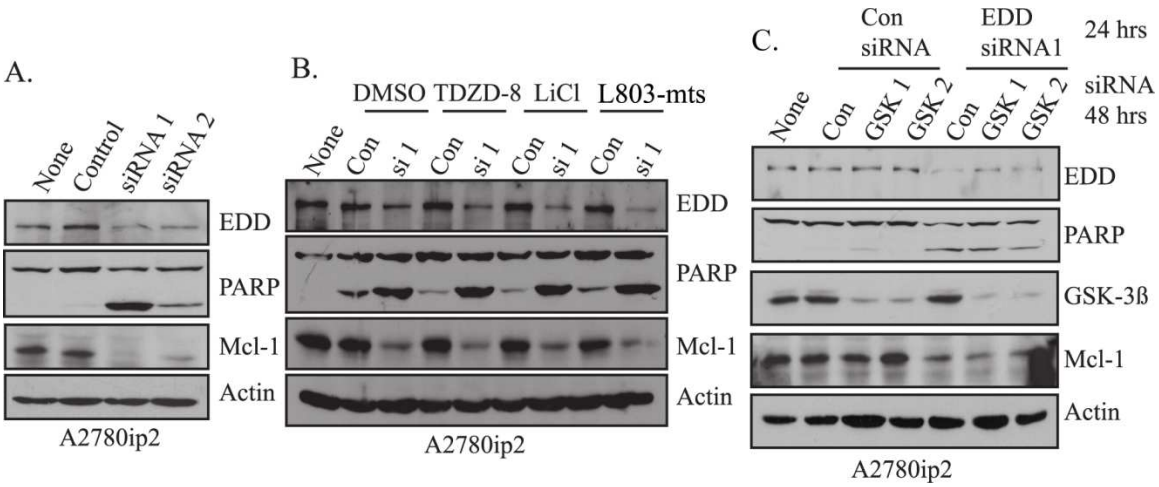


Figure 2.4. EDD regulates Mcl-1 levels through a GSK-3β-independent mechanism. (A) A2780ip2 cells were transfected with control siRNA, EDD siRNA1, or EDD siRNA2 and cell lysates were immunoblotted for EDD, PARP, and Mcl-1. (B) Cells were either untreated (None) or transfected with control (Con) or EDD siRNA1 (si1) and treated with DMSO or the GSK-3β inhibitors TDZD-8, LiCl, or L803-mts. Floating and adherent cells were harvested at 24hr and cell lysates immunoblotted as indicated. (C) A2780ip2 cells were either untreated (None) or transfected with either control siRNA (Con) or either of two GSK-3β siRNAs. After 24hr, the cells were transfected again with either control (Con) or EDD siRNA1. After an additional 24hr, cell lysates from both floating and adherent cells were immunoblotted as indicated.

EDD enhances Mcl-1 expression at the messenger RNA level

The above results suggest that EDD may regulate Mcl-1 synthesis, not its degradation. Indeed, quantitative real-time PCR analysis demonstrated that EDD knockdown inhibited Mcl-1 messenger RNA (mRNA) expression by 1.87-fold in both A2780ip2 and ES-2 cells at 12 and 24hr, respectively, compared with transfection with control siRNA, demonstrating that EDD downregulation inhibits Mcl-1 transcription (Figure 2.5A).

EDD has been shown to act as a transcriptional co-activator for the progesterone and vitamin D receptors, independent of the C-terminal ubiquitin ligase domain (Henderson, Russell et al. 2002). Flag-EDD co-transfection in HeLa cells enhanced transcription from an Mcl-1 promoter-driven luciferase reporter p(−2389/+10)mcl-luc by 5-fold when normalized to cotransfected TK *Renilla* luciferase (Figure 2.5B) (Chao, Wang et al. 1998). Transfection of the ubiquitin ligase-deficient point mutant, Flag-EDD-C2768A, also induced luciferase expression 5-fold. Western blotting confirmed equal EDD expression (Figure 2.5C). Regulation of the Mcl-1 promoter likely occurs at the region 70 to 203 nucleotides preceding the transcriptional start site (Figure 2.5D).These data suggest that EDD positively regulates Mcl-1 transcription, independent of its ubiquitin ligase activity.

Figure 2.5

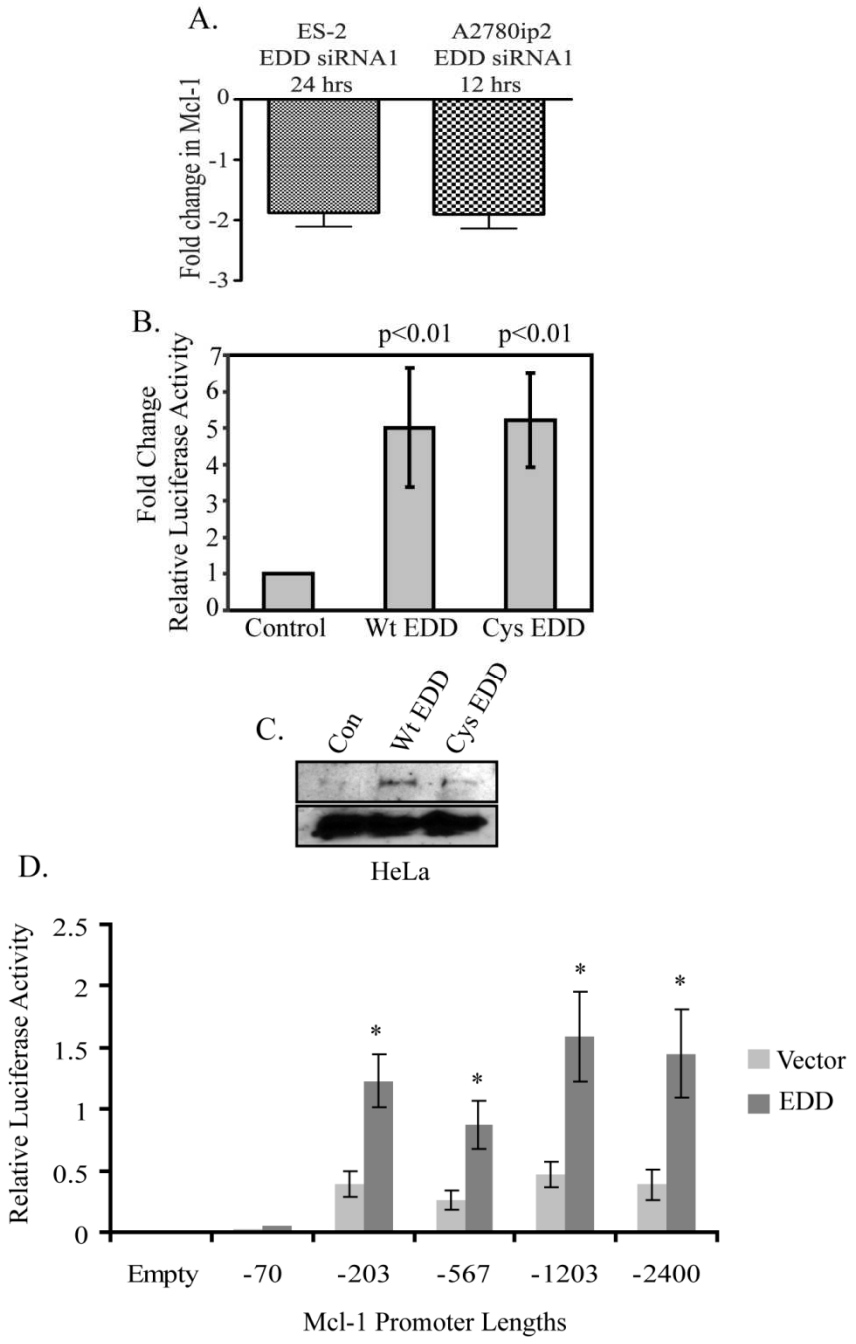

A.
ES-2
EDD siRNA1
24 hrs
A2780ip2
EDD siRNA1
12 hrs
Fold change in Mcl-1
0
-1
-2
-3
B.
p<0.01
p<0.01
Fold Change
Relative Luciferase Activity
7
6
5
4
3
2
1
0
Control
Wt EDD
Cys EDD
C.
Con
Wt EDD
Cys EDD
HeLa
D.
Relative Luciferase Activity
2.5
2
1.5
1
0.5
0
*
*
*
*
Vector
EDD
Empty
-70
-203
-567
-1203
-2400
Mcl-1 Promoter Lengths

Figure 2.5. EDD regulates Mcl-1 levels through transcriptional regulation. (A) EDD knockdown inhibits Mcl-1 mRNA expression. ES-2 and A2780ip2 cells were transfected with EDD siRNA1 for 24 or 12hr, respectively, and RNA was harvested. Quantitative real-time PCR was performed using Mcl-1-specific primers. The *y*-axis represents the fold change in Mcl-1 mRNA in EDD siRNA1-transfected cells compared with that in control siRNA-transfected cells. The results are a combination of three independent experiments. (B) EDD activates the Mcl-1 promoter. HeLa cells were transfected with p(−2389/+10)mcl-luc, an Mcl-1 promoter-driven firefly luciferase plasmid, TK *Renilla* luciferase, and either Flag-EDD, Flag-EDD-C2768A, or empty vector. Cells were harvested at 48hr and firefly luciferase activity was normalized to *Renilla* luciferase activity in each sample. *P* values indicate significance ($P < 0.05$) within a group between Flag-EDD- and vector-transfected cells. These results are a combination of four independent experiments. (C) Western blot of Flag-EDD from (B). (D) As in (B), HeLa cells were transfected with p(−2389/+10)mcl-luc, p(−1289/+10)mcl-luc, p(−567/+10)mcl-luc, p(-70/+10)mcl-luc, or empty mcl-luc, with p(-2389/+10) being the longest Mcl-1 promoter in the luciferase assay. HeLa cells were also transfected with TK *Renilla* luciferase and Flag-EDD or empty vector. After 48hr, luciferase activity was assayed and normalized to *Renilla* luciferase activity. This experiment is a representative experiment out of three experiments. An "*" indicates significance between Flag-EDD and vector-transfected cells ($P<0.05$).

Discussion

This study illustrates that EDD enhances cell survival through the prosurvival protein Mcl-1, an important mediator of survival in ovarian cancer cells (Shigemasa, Katoh et al. 2002, Simonin, Brotin et al. 2009, Brotin, Meryet-Figuiere et al. 2010). EDD knockdown inhibited Mcl-1 mRNA and endogenous protein expression, whereas EDD overexpression increased Mcl-1 transcriptional expression in luciferase assays using the murine Mcl-1 promoter. Induction of the Mcl-1 promoter was independent of EDD's ubiquitin ligase activity, as mutation of the critical cysteine residue in the E3 ligase domain still allowed for induction of the Mcl-1 promoter. This is in agreement with a previous study that showed that EDD acted as a transcriptional co-activator through the middle third of the protein, independent of the C-terminal ubiquitin ligase domain (Henderson, Russell et al. 2002).

Several transcription factors have been demonstrated to regulate Mcl-1 expression, some of which have links to EDD. Platelet-derived growth factor stimulation of prostate cancer cells enhances Mcl-1 expression via a β-catenin and hypoxia-inducible factor 1 alpha subunit-dependent pathway and EDD ubiquitinates β-catenin to promote its stabilization, nuclear localization and activity (Hay-Koren, Caspi et al. 2011, Iqbal, Zhang et al. 2012). E2F transcription factor 1 represses Mcl-1 expression and knockdown of EDD induces E2F transcription factor 1 protein levels in HeLa cells (Croxton, Ma et al. 2002, Munoz, Saunders et al. 2007). Transcription factor software analysis (TFSEARCH) of the human Mcl-1 promoter (accession no. DQ088966) identified

potential binding sites for other transcription factors, including GATAs 1–3, heat shock factors 1 and 2, nuclear factor kappa B and activator protein 1. The progesterone receptor cooperates with GATA-2 in transcriptional activation in breast cancer cells, suggesting that EDD–progesterone receptor interactions may regulate Mcl-1 expression through a GATA-2-dependent pathway (Henderson, Russell et al. 2002, Magklara and Smith 2009). We have not ruled out translational control of Mcl-1 expression by EDD as an additional mechanism of regulation.

Chapter 3

EDD increases cisplatin resistance in ovarian cancer cells

***Note: This chapter contains a portion of the paper:**
***Carcinogenesis.* 2014 May 1; volume 35, number 5, pages 1100-1109.**

EDD enhances cell survival and cisplatin resistance and is a therapeutic target for epithelial ovarian cancer.

Bradley A, Zheng H, Ziebarth A, Sakati W, Branham-O'Connor M, Blumer JB, Liu Y, Kistner-Griffin E, Rodriguez-Aguayo C, Lopez-Berestein G, Sood AK, Landen CN Jr, Eblen ST.

Introduction

Initial therapy for ovarian cancer involves surgical debulking combined with chemotherapy, which consists of platinum and paclitaxel; however, resistance to chemotherapy often occurs in recurrent tumors. Identifying mechanisms of acquired drug resistance is important to developing novel therapeutics. One indicator of poor prognosis in recurrent ovarian cancer is the E3 ubiquitin ligase EDD (E3 ligase identified by differential display) (Clancy, Henderson et al. 2003). E3 ubiquitin ligases modify proteins through the addition of ubiquitin, most often resulting in protein degradation (Wolf and Hilt 2004, Rechsteiner and Hill 2005). EDD contains a C-terminal HECT (Homologous to the E6-AP Carboxyl Terminus) ubiquitin ligase domain and is the human homolog of the *Drosophila* tumor suppressor hyperplastic discs (hyd), which regulates imaginal disk formation (Callaghan, Russell et al. 1998). EDD has a reported role in the DNA damage response and has been implicated in the S phase and G_2/M DNA damage checkpoints (Henderson, Russell et al. 2002, Munoz, Saunders et al. 2007, Gudjonsson, Altmeyer et al. 2012). EDD enhances activation of the DNA damage response kinase Chk2 in response to ionizing radiation or the radiomimetic phleomycin (Henderson, Munoz et al. 2006).

While low in benign tissue and borderline ovarian tumors, EDD is overexpressed in 47% of all types of ovarian cancer and 73% of serous ovarian

tumors (Clancy, Henderson et al. 2003). The EDD protein is also overexpressed or mutated in several solid tumors including breast, hepatocellular, tongue, gastric, and melanoma (Mori, Sato et al. 2002, Clancy, Henderson et al. 2003, Fuja, Lin et al. 2004). EDD is also associated with a 2-fold increased risk of recurrence and death in patients that initially responded to chemotherapy (O'Brien, Davies et al. 2008). The *edd* gene is on chromosome 8q22.3 and amplification of this chromosomal region is associated with cisplatin resistance (Wasenius, Jekunen et al. 1997, Callaghan, Russell et al. 1998). Knockdown of EDD with small interfering RNA (siRNA) decreased colony formation in A2780-cp70 ovarian cancer cells, a derivative selected for cisplatin resistance *in vitro*, when co-treated with cisplatin (O'Brien, Davies et al. 2008). Collectively, these results suggest that EDD may play a role in tumor maintenance and/or cisplatin resistance.

Altered expression of many genes and proteins is reported in tumor tissue and in isogenic cell lines that have been selected for cisplatin resistance. However, many of these studies failed to demonstrate that changes in expression of a particular protein were sufficient to induce cisplatin resistance, raising the possibility that the observed overexpression of EDD in ovarian tumors may not be directly responsible for acquired cisplatin resistance. These results show that EDD directly contributes to cisplatin resistance through its E3 ubiquitin ligase activity in ovarian cancer cells and provides evidence for EDD as a therapeutic target for the treatment of epithelial ovarian cancer.

Materials and Methods

Cell lines and antibodies

ES-2 cells were from Runzhao Li, A2780ip2 cells were from Charles Landen, and OVCAR5 cells were from Thomas Hamilton.COS-7 cells were from American Type Culture Collection (ATCC, Manassas, VA). Stable EDD shRNA cells were generated by retroviral transduction: control shRNA (5′GCTGCAAGACCA TACACTTAT), EDD-shRNA1 (5′GCTGTAGATTTCAACTTAGAT), EDD-shRNA2 (5′GCCATTAGAAAGAACCACAAA) and EDD-shRNA3 (5′TGACAGCAGAACA ACATAATT). Puromycin-resistant clones (ES-2 and A2780ip2) or populations (OVCAR5) were selected. Cisplatin was from Sigma–Aldrich (St Louis, MO). Poly(ADP-ribose) polymerase (PARP) antibody was from Cell Signaling and the EDD (M19) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Lipofectamine 2000 (Invitrogen) was used for transfections, according to the manufacturer's protocol.

siRNA transfection

Cell lines were transfected with 45 nmol of control or EDD siRNA (Sigma–Aldrich). siRNA1: SASI_Hs01_00175227 (5′CCAUUUACCCUGGCUAGUA); siRNA2: SASI_Hs02_00348492 (5′GCGACUCUCCAUGGUUUCU). Control siRNA was Universal Negative Control #1 (Sigma–Aldrich).

Western blotting

Floating and adherent cells were lysed with M2 lysis buffer containing 0.5% sodium dodecyl sulfate (Eblen, Catling et al. 2001). Typically, 65 µg of protein

lysate was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (7–12% gradient gel) and immunoblotted proteins were visualized using enhanced chemiluminescence (Pierce).

Crystal violet staining

Cells were fixed with 4% paraformaldehyde, stained with 0.05% crystal violet in 2% ethanol for 15 min, washed five times with phosphate-buffered saline and dried. Stained cells were solubilized with 2% sodium dodecyl sulfate in phosphate-buffered saline and absorbance was measured at 550 nm.

Flow cytometry

Floating and adherent cells were fixed in ethanol and stained with propidium iodide (Molecular Probes, Eugene, OR). DNA content was determined by flow cytometry and sub-2n cells were counted as apoptotic. The Student's *t*-test was performed on three independent experiments done in duplicate.

MTS assay

Stable ES-2 shRNA cell lines were plated in quadruplicate onto 96-well dishes and treated with cisplatin or saline for 72 h. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) reagent (Promega, Madison, WI) was added for the last 2hr and absorbance measured. The results are a combination of three independent experiments.

Apoptosis assay

COS-7 cells on coverslips were transfected with 2 µg of Flag-EDD, Flag-EDD-C2768A or green florescent protein (GFP). After 24 h, the cells were treated with

cisplatin for 24hr and fixed with 4% paraformaldehyde. Apoptotic cells were labeled using the TACS® 2 Tdt-Blue Label *In Situ* Apoptosis Detection Kit (Trevigen, Gaithersburg, MD). Flag-EDD-transfected cells were immunostained with M2 anti-Flag antibody (Sigma–Aldrich), followed by fluorescein isothiocyanate-labeled secondary antibody (Jackson ImmunoResearch, West Grove, PA). At least 500 transfected cells per coverslip were counted and the percentage of transfected apoptotic cells was determined. Four independent experiments were performed for the cisplatin dose experiment. For the EDD-C2768A experiment, three independent experiments were performed comparing GFP, EDD, and EDD-C2768A at a single dose of 15 μM cisplatin. The data for GFP compared with EDD included the data from the 15 μM group in the cisplatin dose experiment, for an $n = 7$. Two-sample *t*-tests were conducted to determine significance.

Intraperitoneal ovarian cancer model and *in vivo* delivery of siRNA

Female athymic nude mice (NCr-nu) were purchased from the National Cancer Institute (Frederick, MD) after Institutional Animal Care and Use Committee approval of protocols and cared for in accordance with guidelines of the American Association for Accreditation of Laboratory Animal Care. ES-2 and A2780ip2 cells were suspended in serum-free Hanks' balanced salt solution at a concentration of 5×10^6 cells/ml, and 1×10^6 cells were injected intraperitoneally in 200 μl into 40 mice per experiment. After 1 week, mice ($n = 10$ per group) were randomized to treatment with (i) 5 μg control siRNA (sense sequence: 5′-UUCUCCGAACGUGUCACGU-3′, Sigma) in 1,2-dioleoyl-sn-glycero-3-

phophatidylcholine (DOPC), (ii) 5 µg anti-human EDD siRNA (Sigma product SASI_Hs01_00175227), (iii) 5 µg control siRNA plus cisplatin or (iv) 5 µg EDD-targeting siRNA in DOPC plus cisplatin. siRNA constructs were incorporated in DOPC nanoparticles (DOPC) as described previously (Landen, Kinch et al. 2005, Landen, Merritt et al. 2006) and the lyophilized product was stored at −4℃ for <4 weeks. Prior to treatment, the siRNA/DOPC complex was reconstituted in 0.9% saline and administered intraperitoneally twice per week in a volume of 100 µl. Cisplatin was administered intraperitoneally at a dose of 40 µg weekly. Mice were treated for 4 weeks before killing and tumor collection. Tumors were excised and total tumor weight recorded. Statistical analysis comparisons of tumor weights were made using a two-tailed Student's *t*-test, if assumptions of data normality were met. Those represented by alternate distribution were examined using a non-parametric Mann–Whitney *U*-test. Differences between groups were considered statistically significant at $P < 0.05$. Error bars represent standard error. Number of mice per group ($n = 10$) was chosen as directed by a power analysis to detect a 50% decrease in tumor growth with beta error of 0.2. Immunohistochemistry was performed using anti-EDD antibody.

Results

EDD knockdown increases cisplatin sensitivity

O'Brien et al. showed that EDD siRNA reduced colony formation after cisplatin treatment in the cisplatin-resistant A2780-cp70 cell line (O'Brien, Davies et al. 2008). However, although 72hr cisplatin treatment induced dose-dependent cell death in ES-2 and A2780ip2 cells transfected with control siRNA (Figure 3.1A and B), the catastrophic apoptosis induced by EDD siRNA obscured any cisplatin effect. At 24hr of cotreatment, EDD knockdown in ES-2 cells conferred cisplatin sensitivity (Figure 3.1C), whereas the strong apoptotic response of EDD knockdown alone in A2780ip2 cells masked any potential effects on cisplatin sensitization (Figure 3.1D). Although EDD knockdown induced apoptosis in A2780-cp20 cisplatin-resistant cells, it did not enhance cell death in response to cisplatin in these cells (Figure 3.1E).

In order to separate the basic cell survival function of EDD from a potential role in cisplatin resistance, we generated ES-2 (Figure 3.2A), A2780ip2 (Figure 3.2B) and OVCAR5 (Figure 3.2C) cell lines with constitutive knockdown of EDD using retroviral transduction of three separate shRNAs. These cells represent the small portion of the population that can survive initial EDD knockdown, as the majority of the cells undergo apoptosis. Immunoblotting showed that these pools of cells survive because they are not dependent upon EDD for Mcl-1 expression (Figure 3.3). Cellular clones of ES-2 and A2780ip2 cells and a population of OVCAR5 cells were selected. MTS assays demonstrated that ES-2 clones expressing EDD shRNA were 4- to 21-fold more sensitive to cisplatin than cells

expressing control shRNA, with EC_{50} values of 48.8 μM for the control-1 (clone 1) shRNA line, 12.0 μM for EDD shRNA1, 7.4 μM for EDD shRNA2, and 2.3 μM for the EDD shRNA3 cell lines (Figure 3.2D). In addition, A2780ip2 (Figure 3.2E) and OVCAR5 (Figure 3.2F) EDD shRNA cells were more sensitive to cisplatin after 24hr of treatment compared with the control shRNA cells, as measured by increased induction of PARP cleavage. These results demonstrate that stable loss of EDD sensitizes cells to cisplatin.

Figure 3.1

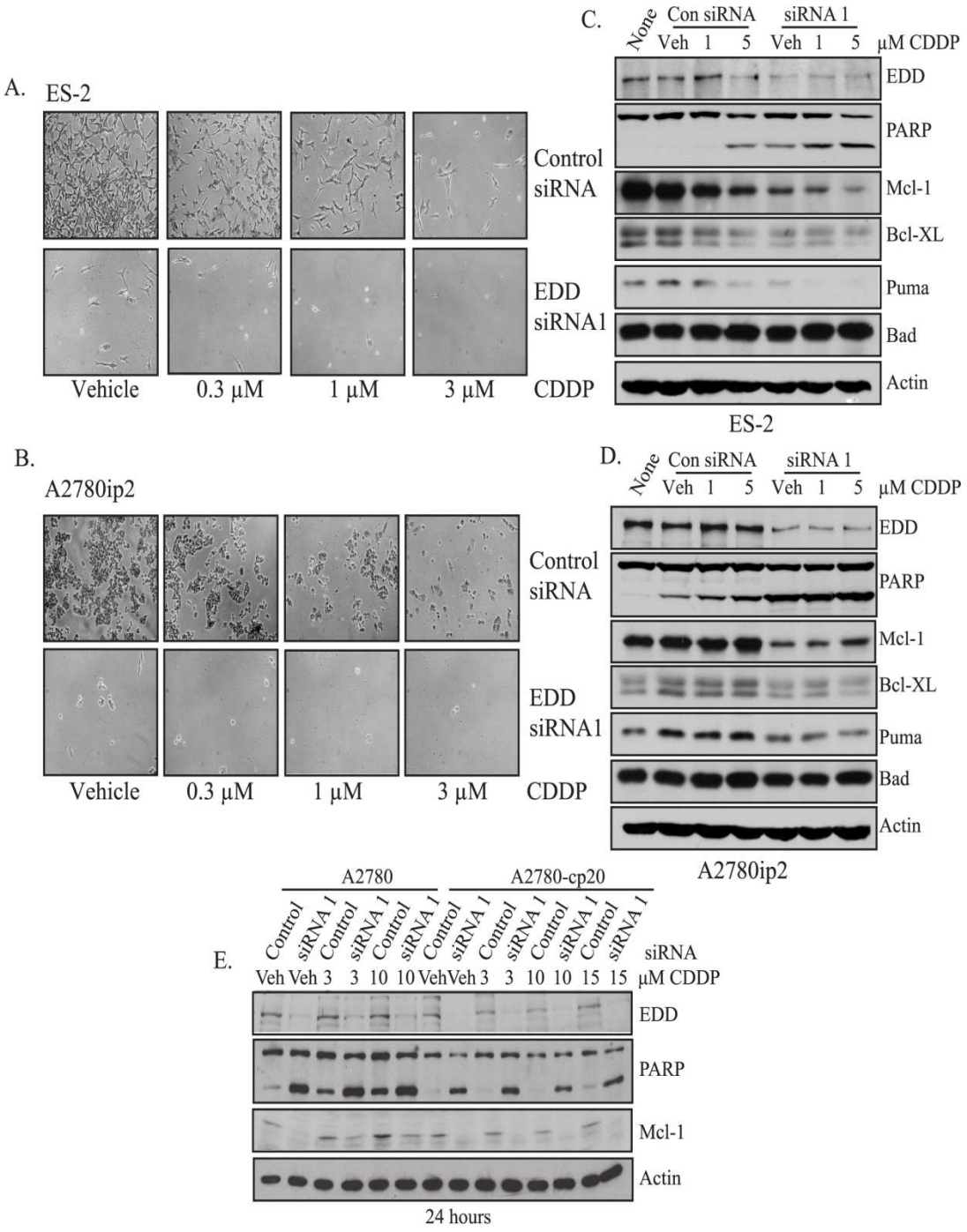

A.
ES-2
Control siRNA
EDD siRNA1
Vehicle
0.3 μM
1 μM
3 μM
CDDP
B.
A2780ip2
Control siRNA
EDD siRNA1
Vehicle
0.3 μM
1 μM
3 μM
CDDP
C.
None
Con siRNA
siRNA 1
Veh 1 5 Veh 1 5 μM CDDP
EDD
PARP
Mcl-1
Bcl-XL
Puma
Bad
Actin
ES-2
D.
None
Con siRNA
siRNA 1
Veh 1 5 Veh 1 5 μM CDDP
EDD
PARP
Mcl-1
Bcl-XL
Puma
Bad
Actin
A2780ip2
E.
A2780
A2780-cp20
Control siRNA 1 Control siRNA 1 Control siRNA 1 Control siRNA 1 Control siRNA 1 Control siRNA 1
siRNA
Veh Veh 3 3 10 10 Veh Veh 3 3 10 10 15 15 μM CDDP
EDD
PARP
Mcl-1
Actin
24 hours

Figure 3.1. Transient EDD knockdown may sensitize cells to cisplatin. (A) ES-2 and (B) A2780ip2 cells were transfected with either control siRNA or EDD siRNA1 and immediately treated with vehicle, 0.3, 1, or 3 μM cisplatin for 72hr. Photographs were taken at 10X magnification. (C) ES-2 and (D) A2780ip2 cells were either untreated (None) or transfected with either control siRNA or EDD siRNA1. Cells were treated with Vehicle (Veh), 1, or 5 μM cisplatin immediately after transfection and harvested at 24hr for immunoblotting. (E) Apoptosis is induced in cisplatin resistant cells upon EDD knockdown. A2780 and the cisplatin-resistant A2780-cp20 cell line were transfected with control siRNA or EDD siRNA1 and then treated with increasing amounts of cisplatin. Cells were harvested at 24hr and immunoblotted as indicated.

Figure 3.2

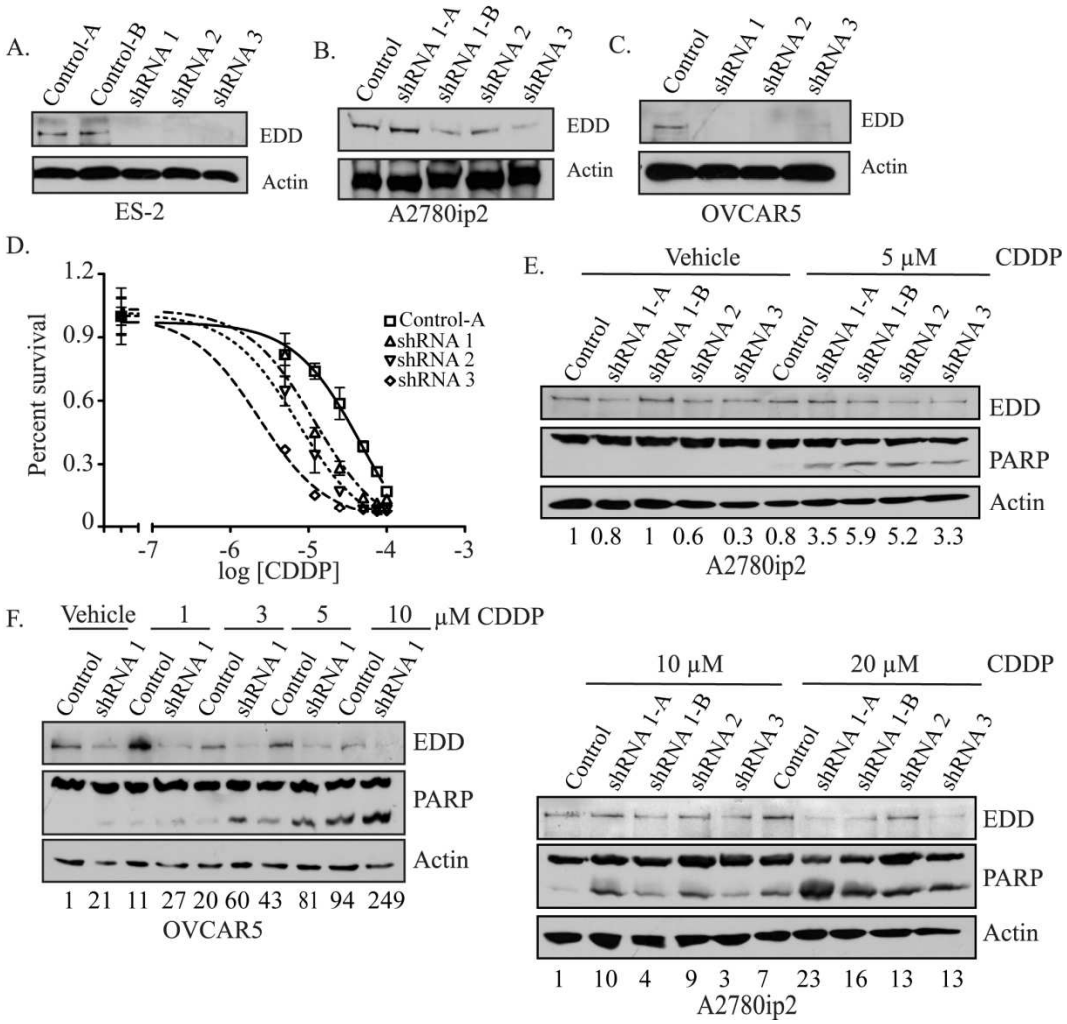

A.
Control-A
Control-B
shRNA 1
shRNA 2
shRNA 3
EDD
Actin
ES-2
B.
Control
shRNA 1-A
shRNA 1-B
shRNA 2
shRNA 3
EDD
Actin
A2780ip2
C.
Control
shRNA 1
shRNA 2
shRNA 3
EDD
Actin
OVCAR5
D.
Percent survival
1.2
0.9
0.6
0.3
0
-7
-6
-5
-4
-3
log [CDDP]
Control-A
shRNA 1
shRNA 2
shRNA 3
E.
Vehicle
5 μM
CDDP
Control
shRNA 1-A
shRNA 1-B
shRNA 2
shRNA 3
EDD
PARP
Actin
1 0.8 1 0.6 0.3 0.8 3.5 5.9 5.2 3.3
A2780ip2
F.
Vehicle
1
3
5
10
μM CDDP
Control
shRNA 1
EDD
PARP
Actin
1 21 11 27 20 60 43 81 94 249
OVCAR5
10 μM
20 μM
CDDP
Control
shRNA 1-A
shRNA 1-B
shRNA 2
shRNA 3
EDD
PARP
Actin
1 10 4 9 3 7 23 16 13 13
A2780ip2

Figure 3.2. Stable EDD knockdown increases cisplatin sensitivity. (A) ES-2, (B) A2780ip2 and (C) OVCAR5 cells were retrovirally transduced with control or one of three EDD shRNAs and clones (ES-2 and A2780ip2) or populations (OVCAR5) were selected. Cell lysates were immunoblotted for EDD expression. Multiple clones from the same shRNA are designated as A or B. (D) ES-2 control shRNA or EDD shRNA cells were treated with cisplatin for 72h and cell viability measured by MTS assay. Percent survival was plotted against the log of the cisplatin concentration. The results are from three independent experiments performed in quadruplicate. (E) A2780ip2 and (F) OVCAR5 shRNA cells were treated with cisplatin for 24hr and cell lysates from floating and adherent cells were immunoblotted for EDD and PARP cleavage. The numbers underneath the blot represent the relative intensity of cleaved PARP in each lane compared with the first lane of each blot.

Figure 3.3

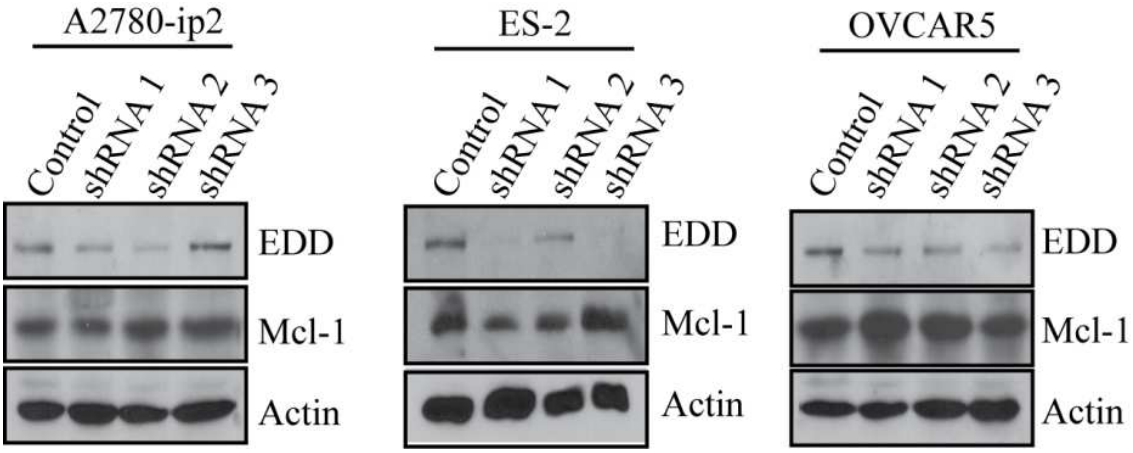


Figure 3.3. Normal Mcl-1 expression in EDD stable knockdowns. A2780ip2, ES-2, and OVCAR5 stable knockdown cell lines were immunoblotted for EDD, Mcl-1, and actin.

EDD is sufficient to induce cisplatin resistance

To determine if EDD is sufficient to induce cisplatin resistance, COS-7 cells were transfected with Flag-EDD or GFP for 24hr and then treated with cisplatin for an additional 24hr. Cells were immunostained for Flag-EDD and costained with the TACS® 2 Tdt-Blue Label *In Situ* Apoptosis Detection Kit, staining apoptotic nuclei black under brightfield microscopy (Figure 3.4A). The percentage of transfected cells that were apoptotic after cisplatin treatment was determined by counting. Cells transfected with Flag-EDD had significantly less apoptosis at the higher cisplatin doses of 15 μM (GFP = 8.9%, EDD = 4.0%, $P < 0.03$) and 30 μM (GFP = 14.6%, EDD = 6.0%, $P < 0.02$) compared with the GFP-transfected cells, demonstrating that EDD overexpression was sufficient to induce cisplatin resistance (Figure 3.5A). To determine if EDD ubiquitin ligase activity was required, cells were transfected with GFP, Flag-EDD, or Flag-EDD-C2768A, a ubiquitin ligase-deficient mutant, and treated with 15 μM cisplatin for 24hr. EDD-C2768A did not induce cisplatin resistance compared with the GFP control, whereas EDD caused 2.4-fold protection (GFP = 9.4%, EDD = 3.8%, EDD-C2768A = 11.8%) (Figure 3.5B). Statistical significance was seen between EDD and GFP, and EDD and EDD-C2768A. These results show that EDD-induced cisplatin resistance is dependent upon its E3 ubiquitin ligase activity. EDD localizes to the nucleus, where cisplatin induces DNA damage, and mutation of EDD at Cys2768 did not affect nuclear localization (Figure 3.4B) (Henderson, Russell et al. 2002, Henderson, Munoz et al. 2006).

Figure 3.4

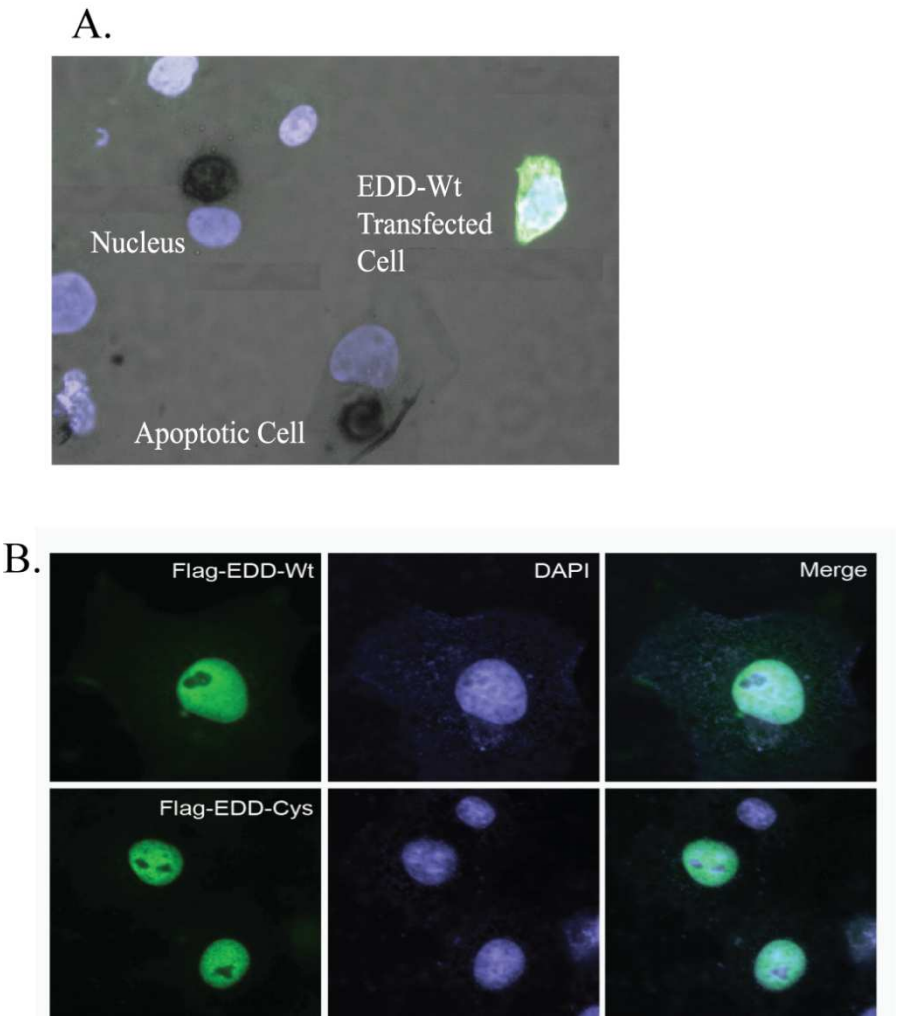


Figure 3.4. EDD overexpression in apoptosis assay and EDD localization. (A) COS-7 cells plated on coverslips were transfected with Flag-EDD (24hr) and then treated with cisplatin for an additional 24hr. Cells were fixed and stained for the transfected gene (green) and apoptotic (black) cells and DAPI (blue). (B) EDD mutation does not affect nuclear localization. COS-7 cells on coverslips were transfected with either Flag-EDD or Flag-EDD-C2768A. The cells were then fixed and immunostained with anti-Flag antibody followed by FITC-labeled secondary antibody and DAPI stained.

Figure 3.5

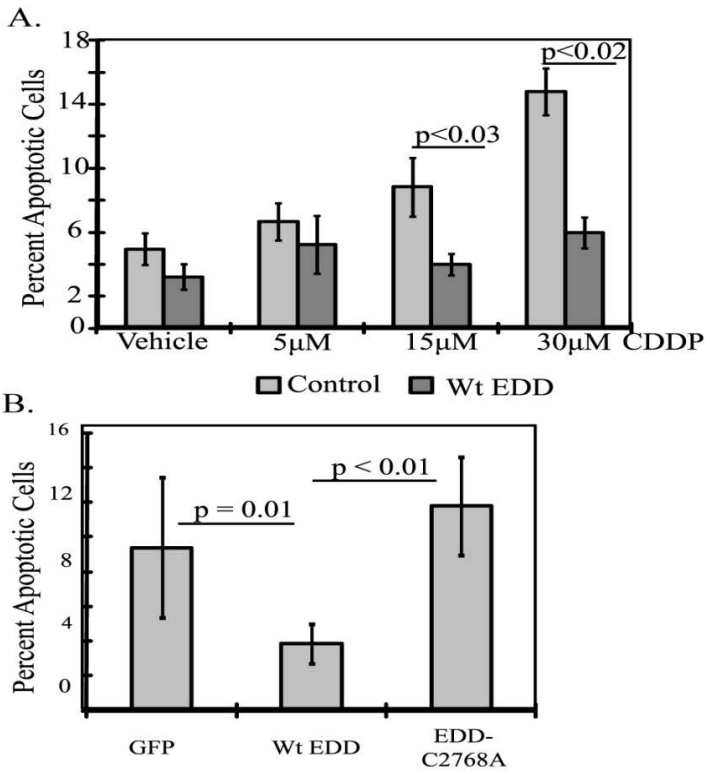


Figure 3.5. EDD overexpression is sufficient to induce cisplatin resistance, dependent upon its ubiquitin ligase activity. (A) COS-7 cells on coverslips were transfected with Flag-EDD or GFP for 24hr and then treated with cisplatin for an additional 24hr. Fixed cells were stained for transfected and apoptotic cells and 4′,6-diamidino-2-phenylindole stained, as shown in Figure 3.4. The percentage of apoptotic transfected cells was determined by cell counting. At least 500 cells were counted per condition in each of four experiments. (B) Same as in (A), but cells were transfected with GFP, Flag-EDD or Flag-EDD-C2768A, an ubiquitin ligase-deficient mutant. Cells were treated with 15 μM cisplatin for 24hr on the day following transfection and the percentage of apoptotic transfected cells was determined by cell counting 24hr later.

EDD knockdown *in vivo* enhances cisplatin efficacy

Charles Landen, Jr. and Anil Sood have previously demonstrated *in vivo* delivery of siRNA to ovarian tumors via DOPC liposomal nanoparticles, resulting in knockdown of the target protein and a reduction in tumor burden (Landen, Kinch et al. 2005, Halder, Kamat et al. 2006, Landen, Merritt et al. 2006, Lin, Immaneni et al. 2008, Merritt, Lin et al. 2008, Mangala, Han et al. 2009, Chakravarty, Roy et al. 2011, Nick, Stone et al. 2011). To determine if EDD is a viable target for the treatment of ovarian cancer, we generated intraperitoneal xenografts of ES-2 and A2780ip2 cells in female athymic nude mice. One week later, 10 mice per group were treated intraperitoneally twice per week with either control or EDD siRNA1 in DOPC liposomes, in combination with either cisplatin or saline treatment once weekly. After 4 weeks, mice were killed and tumor tissue was harvested. When compared to control siRNA treatment alone, cisplatin combined with control siRNA/DOPC showed a trend toward significance in ES-2 xenografts when measuring tumor weight (37.7% reduction, $P = 0.167$) but became statistically significant when cisplatin was combined with EDD siRNA1/DOPC (77.9% reduction, $P = 0.004$) (Figure 3.6A). In A2780ip2 xenografts, cisplatin plus control siRNA/DOPC treatment was not significantly different compared with control siRNA/DOPC alone (39.2% reduction, $P = 0.349$), but cisplatin plus EDD siRNA1/DOPC was significantly better than control siRNA/DOPC alone (75.9% reduction, $P = 0.042$). In those mice treated with cisplatin, cotreatment with EDD siRNA1/DOPC was significantly better than co-treatment with control siRNA/DOPC in ES-2 (64% reduction, $P = 0.035$) and

showed a trend toward significance in A2780ip2 (60.3% reduction, P = 0.168). Immunohistochemistry of A2780ip2 tumors with EDD antibody showed EDD expression in tumors treated with control siRNA, with a possible enhancement of EDD expression in tumors following cisplatin treatment (Figure 3.6B and C). EDD siRNA1 treatment *in vivo* decreased EDD expression in tumors (Figure 3.6D and E). Collectively, these results suggest that therapies targeting EDD expression might be an attractive treatment for ovarian cancer patients.

Figure 3.6

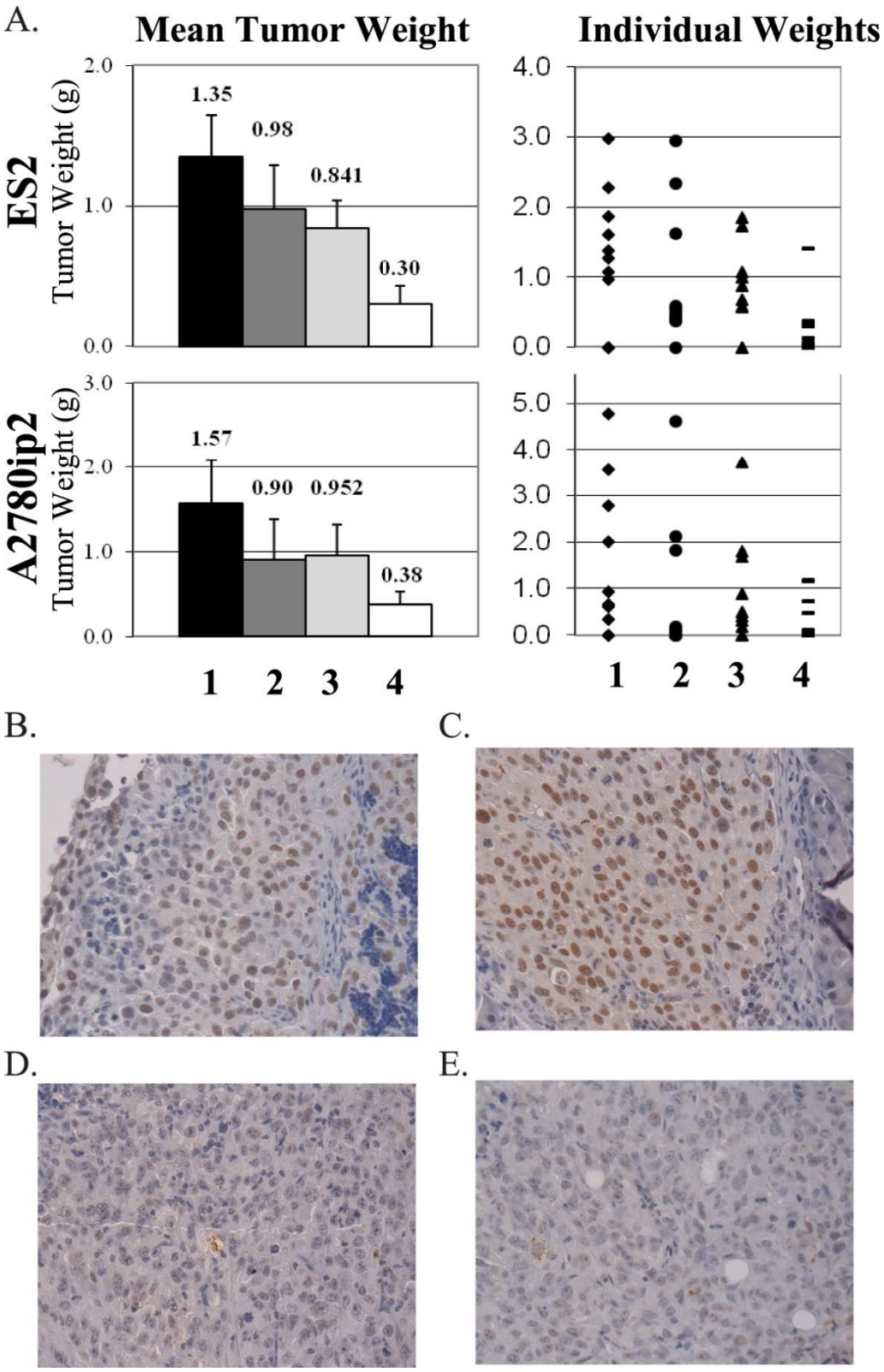

A.
Mean Tumor Weight
Individual Weights
ES2
Tumor Weight (g)
1.35
0.98
0.841
0.30
A2780ip2
Tumor Weight (g)
1.57
0.90
0.952
0.38
1
2
3
4
B.
C.
D.
E.

Figure 3.6. DOPC nanoparticle delivery of EDD siRNA *in vivo* reduces tumor burden. (A) ES-2 or A2780ip2 cells were injected intraperitoneally into 40 female athymic nude mice per cell line. Mice were either treated with control siRNA in DOPC (lane 1), EDD siRNA1 in DOPC (lane 2), control siRNA in DOPC plus cisplatin (lane 3), or EDD siRNA1 in DOPC plus cisplatin (lane 4). Mice were treated for 4 weeks before killing and tumor collection. Tumors were excised and total tumor weight determined. The number above each lane represents the mean tumor weight in grams. Immunohistochemistry demonstrates EDD knockdown *in vivo*. A2780ip2 tumors from mice treated with (B) control siRNA in DOPC, (C) control siRNA in DOPC plus cisplatin, (D) EDD siRNA1 in DOPC and (E) EDD siRNA1 in DOPC plus cisplatin were immunostained with EDD antibody followed by horseradish peroxidase secondary antibody.

Discussion

These results show that EDD directly regulates cisplatin sensitivity. A previous study has shown that EDD overexpression correlates with poor survival for patients with recurrent ovarian cancer and that knockdown of EDD with siRNA in cisplatin-resistant A2780-cp70 cells decreases colony formation by 40% after cisplatin treatment (O'Brien, Davies et al. 2008). However, a portion of this effect may be due to the cell survival functions of EDD described in the previous chapter. To separate these functions, we generated stable knockdown cells to select for those cells that could survive initial EDD knockdown. These cells showed normal levels of Mcl-1, demonstrating that this small portion of the initial cell population was not dependent upon EDD for Mcl-1 expression. By separating these functions, we demonstrated that loss of EDD sensitizes cells to cisplatin. Expression of EDD in ovarian cancer cell lines does not directly correlate with reported cisplatin sensitivity, as some ovarian cancer cell lines with high EDD expression have low cisplatin IC_{50}s and some of those with higher resistance express lower levels of EDD (Figure 2.1 and Table 3.1) (Smith, Ngo et al. 2005, Matsumura, Huang et al. 2011, Ye, Fu et al. 2011, Saran, Arfuso et al. 2012). This is likely due to the multiple mechanisms of cisplatin resistance in cells and tumors (Galluzzi, Senovilla et al. 2012). Indeed, A2780-cp70 cells selected *in vitro* for cisplatin resistance after long-term exposure did not have higher levels of EDD expression than parental A2780 cells (O'Brien, Davies et al. 2008). Importantly, we show that overexpression of EDD was sufficient to induce resistance to cisplatin and was dependent upon EDD ubiquitin ligase activity.

EDD has been suggested to play a role in the DNA damage response, particularly in response to double strand breaks. EDD and the E3 ubiquitin ligase TRIP12 regulate levels of RNF168, a regulator of histone ubiquitination after DNA damage, resulting in controlled spread of histone ubiquitination from the area of double strand breaks (Gudjonsson, Altmeyer et al. 2012); however, no reports have linked RNF168 to cisplatin resistance. EDD is important in activation of the DNA damage response kinase Chk2, as EDD-depleted cells show reduced activation of Chk2 in response to double strand breaks (Henderson, Munoz et al. 2006). EDD knockdown increased sensitivity of HeLa cells to phleomycin, regulating both the S phase and the G_2/M phase checkpoints in treated cells (Henderson, Munoz et al. 2006, Munoz, Saunders et al. 2007, Benavides, Chow-Tsang et al. 2013). In the presence of DNA damage, EDD knockdown cells underwent radio-resistant DNA synthesis and premature entry into mitosis, leading to mitotic catastrophe (Munoz, Saunders et al. 2007).

Both Bcl-xL and Mcl-1 have been implicated to protect ovarian cancer cells from chemotherapy-induced apoptosis, suggesting that EDD upregulation of Mcl-1 expression (as described in the previous chapter) may also contribute to cisplatin resistance; however, the requirement for ubiquitin ligase activity for cisplatin resistance, but not for induction of the Mcl-1 promoter, strongly suggests that the regulation of Mcl-1 by EDD is distinct from the induction of cisplatin resistance as illustrated in Figure 3.7 (Simonin, Brotin et al. 2009). Interestingly, EDD itself appeared to be upregulated in xenografts from mice treated with cisplatin, which may be clinically important in regards to a study showing EDD

overexpression in recurrent ovarian tumors from patients who had a favorable response to initial chemotherapy (O'Brien, Davies et al. 2008).

Small molecule inhibitors of ubiquitin ligases have had little success due to the lack of a defined catalytic domain and the utilization of protein–protein interactions in order to ubiquitinate targets. Charles Landen, Jr. and Anil Sood have previously demonstrated that DOPC nanoparticles can be utilized to efficiently deliver siRNA to ovarian tumor tissue to inhibit tumor growth and metastasis and to enhance chemosensitivity (Landen, Kinch et al. 2005, Halder, Kamat et al. 2006, Landen, Merritt et al. 2006, Lin, Immaneni et al. 2008, Merritt, Lin et al. 2008, Mangala, Han et al. 2009, Chakravarty, Roy et al. 2011, Nick, Stone et al. 2011). Our *in vivo* data demonstrated that EDD is a valid target for treating epithelial ovarian cancer in combination with chemotherapy. EDD siRNA showed enhanced efficacy over cisplatin treatment alone in ES-2 xenografts and a trend toward significance in A2780ip2 xenografts. This effect of EDD siRNA was likely due to both the positive effects of EDD on cell survival and the enhancement of cisplatin resistance. Upon knockdown *in vivo*, loss of EDD likely enhances both cell death and cisplatin sensitivity. Our findings that EDD regulates survival Mcl-1 regulation independent of its ubiquitin ligase activity and cisplatin resistance through its ubiquitin ligase domain suggest that therapies targeting EDD expression, such as EDD siRNA in nanoparticles, may prove to be a more beneficial therapeutic approach than a chemical inhibitor of EDD ubiquitin ligase activity, although the latter alone may have some beneficial role in enhancing cisplatin sensitivity.

Figure 3.7

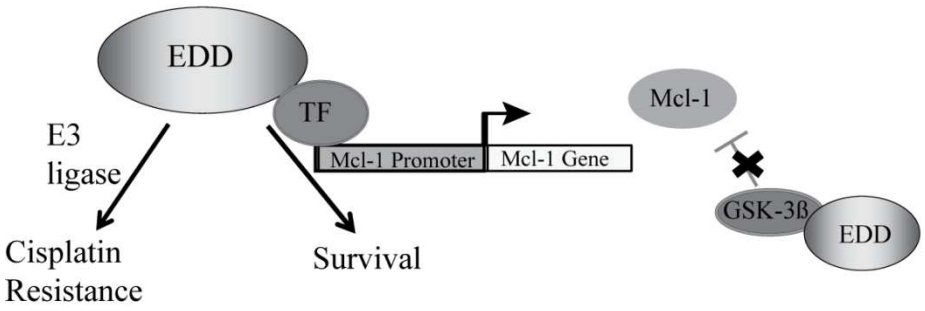


Figure 3.7. Model for EDD regulation of survival and cisplatin resistance. EDD enhances cell survival by promoting Mcl-1 transcriptional expression through regulation of an unknown transcription factor (TF). Enhancement of Mcl-1 protein expression is independent of GSK-3β inhibition of Mcl-1 protein levels. EDD also increases cisplatin resistance through its E3 ubiquitin ligase function.

Table 3.1

Ovarian Cell Line	IC_{50} Cisplatin (μM)	Reference
IOSE	3.17	(Saran, Arfuso et al. 2012)
A2780	1.74	(Matsumura, Huang et al. 2011)
ES-2	48.8	(Bradley, Zheng et al. 2014)
OV2008	1.72	(Ye, Fu et al. 2011)
OVCAR3	25.7	(Smith, Ngo et al. 2005)
OVCAR5	5.02	(Matsumura, Huang et al. 2011)
SKOV-3	21.7	(Smith, Ngo et al. 2005)
TOV-21G	18.5	(Smith, Ngo et al. 2005)

Table 3.1. Cisplatin sensitivities across ovarian cancer cell lines. The reported IC_{50} to cisplatin of the cell lines used in this study and the references they are from. The number from ES-2 cells was derived from our data (Figure 3.2).

Chapter 4

EDD regulates cisplatin resistance in oral squamous cell carcinoma

Introduction

Oral squamous cell carcinoma is diagnosed in more than 40,000 Americans each year, with 20% of these patients ultimately dying from this disease (Siegel, Naishadham et al. 2013). Oral cancer is of primary concern worldwide since it affects over 330,000 people each year with 54% of these patients eventually dying from this type of cancer (Parkin, Bray et al. 2005). Even though most oral squamous cell carcinomas remain localized, some patients experience metastasis to the lymph nodes, which is often difficult to treat even after surgery and radiation. To combat this issue, patients with metastasis are typically treated with chemotherapeutic drugs such as cisplatin, paclitaxel, and 5-fluorouracil. Drug resistance in this type of carcinoma is a reality, but is not highly studied.

The E3 ubiquitin ligase EDD is genetically amplified in 50% of oral squamous cell carcinomas of the tongue (Clancy, Henderson et al. 2003). This is similar to ovarian cancer where *edd* is upregulated in 47% of ovarian cancers (73% of serous ovarian cancers) and this cancer is also commonly treated with cisplatin often leading to drug resistance (Clancy, Henderson et al. 2003). My previous research in ovarian cancer demonstrated that EDD is sufficient to promote cisplatin resistance in ovarian cancer, which is dependent on its ubiquitin ligase activity, as described in Chapter 3 and in my recent publication

(Bradley, Zheng et al. 2014). Based on this research, this led me to hypothesize that EDD may also be involved in mediating cisplatin resistance in oral squamous cell carcinoma since both oral cancer and ovarian cancers are treated with cisplatin and *edd* is upregulated in both.

EDD ubiquitinates several proteins to regulate their degradation (TopBP1, Paip2, Katanin p60, TERT, and RNF168) or stability (βcatenin) (Honda, Tojo et al. 2002, Yoshida, Yoshida et al. 2006, Maddika and Chen 2009, Gudjonsson, Altmeyer et al. 2012, Jung, Wang et al. 2013, Okamoto, Bartocci et al. 2013). It is uncertain at this time which known or unknown ubiquitination targets of EDD are involved in regulating cisplatin resistance in ovarian cancer. Although a connection has not been established between EDD and oral squamous cell carcinoma, overexpression of EDD is correlated with a two-fold increased risk of disease recurrence in ovarian cancer patients that initially responded to chemotherapeutic treatment (O'Brien, Davies et al. 2008). My aim is to establish a connection between EDD and oral squamous cell carcinoma to determine if EDD regulates cisplatin sensitivity in these cells as well as to determine the mechanism by which this could be occurring.

Materials and Methods

Cell lines and antibodies

IOSE cells were from Nelly Auersperg. HeLa and SKOV-3 cells were from American Type Culture Collection (ATCC, Manassas, VA). UM-SCC-9 and UM-SCC-25 cell lines were obtained from Steve Rosenzweig (MUSC). UM-SCC-11A, UM-SCC-11B, UM-SCC-22B, and UM-SCC-74B cells were from Viswanathan (Visu) Palanisamy (MUSC). Inducible stable EDD knockdown cell lines were generated by retroviral transduction with pTRIPZ shRNA (Thermo Scientific, Waltham, MA): Scrambled control (RHS4743), EDD-shRNA1 (V2THS_202102, 5'TAGAGGAATAGAGTGGGAC), EDD-shRNA2 (V2THS_203176, 5'TTGGAA TCTACATTCACTG), EDD-shRNA3 (V2THS_75176, 5'TTATTAAAGAATGCAC ACC). Initial transfection of 3µg shRNA plasmid, 3µg Δ8.91, and 1.5µg pVSV-G were performed with calcium phosphate transfection according to the manufacturer's protocol (Clontech, Mountain View, CA). Stable populations were selected with puromycin and shRNA expression was induced with doxycycline (Sigma-Aldrich, St Louis, MO). Non-inducible stable EDD shRNA cells were generated by retroviral transduction: control shRNA (5′GCTGCAAGACCATACACTTAT), EDD-HP1 (5′GCTGTAGATTTCAACT TAGAT), EDD-HP2 (5′GCCATTAGAAAGAACCACAAA) and EDD-UTR (5′TGACAGCAGAACAACATAATT). Puromycin-resistant populations were selected. Cisplatin was from Sigma–Aldrich (St Louis, MO). Poly (ADP-ribose) polymerase (PARP) antibody was from Cell Signaling and the EDD (M19) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

siRNA transfection

Cell lines were transfected with 45 nmol of control or EDD siRNA (Sigma–Aldrich). siRNA1: SASI_Hs01_00175227 (5′CCAUUUACCCUGGCUAGUA); siRNA2: SASI_Hs02_00348492 (5′GCGACUCUCCAUGGUUUCU). Control siRNA was Universal Negative Control #1 (Sigma–Aldrich).

Western blotting

Floating and adherent cells were lysed with M2 lysis buffer containing 0.5% sodium dodecyl sulfate (Eblen, Catling et al. 2001). Typically, 65 µg of protein lysate was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (7–12% gradient gel) and immunoblotted proteins were visualized using enhanced chemiluminescence (Pierce).

Fluorescence Activated Cell Sorting

Inducible stable EDD knockdown cell lines utilize pTRIPZ shRNA which expresses red fluorescent protein (RFP). Cells were sorted for the top 25% of cells expressing the highest levels of RFP. Cells were treated with doxycycline (0.5µg/mL) daily for one week. Cells ($5x10^6$) were trypsinized, centrifuged at 1500rpm for 5 minutes, washed with 1xPBS, and centrifuged again. Cells were resuspended in 1mL PBS and 1µL of violet LiveDead (Invitrogen) was added, cells incubated on ice for 30 minutes in the dark. Cells were then centrifuged, washed with PBS, centrifuged, and resuspended in 500uL Cell Staining Buffer (PBS + 1%BSA). Finally, cells were filtered through 40uM capped FACS tubes (BD 352235, BD Biosciences, San Jose, CA) just before being sorted on the FACS Aria Iiu Cell Sorter in the Flow Cytometry core at MUSC at RFP

wavelength (553-574nm) and LiveDead violet (405 to 451nm). Cells were sorted into media +50% FBS +Pen/Strep antibiotic and kept on ice until being centrifuged, resuspended in media +10% FBS +Pen/Strep +doxycycline +puromycin, and plated.

Colony Formation Assay

One thousand cells were plated on a 35mm dish. The following day, cells were treated with cisplatin for 2 hours, washed with PBS, and incubated with fresh media +10% FBS for 3-7 days. Cells were collected by being washed twice with PBS and fixed with 4% paraformaldehyde for 20 minutes at room temperature, washed with PBS, stained with 0.05% crystal violet in 2% ethanol for 15 min, washed five times with phosphate-buffered saline, and dried. Pictures were taken of the plates and colonies were counted either manually or with the GelCount Colony Counter (Oxford Optronix, Abingdon, United Kingdom). Stained cells were solubilized with 2% sodium dodecyl sulfate in phosphate-buffered saline and absorbance was measured at 550 nm. For proliferation assays, cells were collected daily for one week without exposure to cisplatin, stained with crystal violet, and solubilized in 2% SDS as described above.

MTS assay

Stable ES-2 shRNA cell lines were plated in quadruplicate onto 96-well dishes and treated with cisplatin or saline for 72hr. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) reagent (Promega, Madison, WI) was added for the last 2hr and absorbance measured. The results are a representation of one experiment.

Results

Transient knockdown of EDD causes apoptosis in oral squamous cell carcinoma cell lines

Based on research from Clancy et al., *edd* is upregulated in 50% of oral squamous cell carcinomas of the tongue (Clancy, Henderson et al. 2003). To determine EDD protein expression in oral squamous cell carcinomas, cell lysates were collected from five different oral squamous cell carcinoma cell lines and compared to protein expression in SKOV3, an ovarian cancer cell line, and IOSE, an immortalized ovarian surface epithelium cell line as shown in Figure 4.1A. While IOSE represents a benign ovarian cell line, a benign oral cell line was not available for comparison. The UM-SCC-9 cell line is a squamous cell carcinoma of the anterior tongue from a 25 year old male. UM-SCC-25 cells are cells from a neck metastasis that initiated in the larynx of a 70 year old male. UM-SCC-11A cells are from an epiglottis tumor in a male, while UM-SCC-11B cells are from a cervical lymph node tumor that formed from this primary tumor. UM-SCC-74A cells were isolated from a tumor at the base of the tongue from a male patient. UM-SCC-74B cells were isolated from the larynx as the metastatic tumor cells from UM-74A cells (Brenner, Graham et al. 2010). The EDD protein is highly expressed in the oral squamous cell carcinoma cell lines analyzed in comparison to the ovarian cell lines (Figure 4.1A). To establish if EDD is essential in these cell lines, cells were transfected with control siRNA or one of two different EDD siRNAs to knockdown EDD expression. EDD siRNA1 has the best ability to knockdown EDD protein expression and was found in UM11B and UM74B cell

lines to promote apoptosis, as indicated by the cleavage of the PARP protein (Figure 4.1B). As seen in ovarian cancer cells, as described in Chapter 2, EDD knockdown causes a decrease in the protein expression of the anti-apoptotic protein Mcl-1 as well, which mediates apoptosis in these cells. Apoptosis can occur in as little as 48 hours after transient EDD knockdown in UM74B cells (Figure 4.1C).

Repeating this experiment with transient knockdown of EDD with siRNA using EDD siRNA1 in SCC9, SCC25, UM11A, UM11B, and UM74B cells, we demonstrated that knockdown of EDD causes apoptosis in SCC25 and UM11B cell lines as shown in Figure 4.1D. As in Figure 4.1B, EDD transient knockdown does not cause apoptosis in UM11A cells. In this experiment, UM74B cells did not undergo efficient knockdown of EDD protein, so a conclusion about sensitivity to EDD knockdown induced apoptosis cannot be made. Changes in Mcl-1 protein expression as a result of EDD knockdown were not consistent, in contrast to those changes seen in Figure 4.1B&C. This indicates that the anti-apoptotic protein Mcl-1 may not be required for mediating cellular survival in all oral squamous cell carcinoma cell lines. SCC9 cells, like UM11A cells, are not sensitive to EDD knockdown induced apoptosis implying that not all of these oral squamous cell carcinoma cell lines utilize the same cell signaling pathways to regulate cell functions such as survival.

Figure 4.1

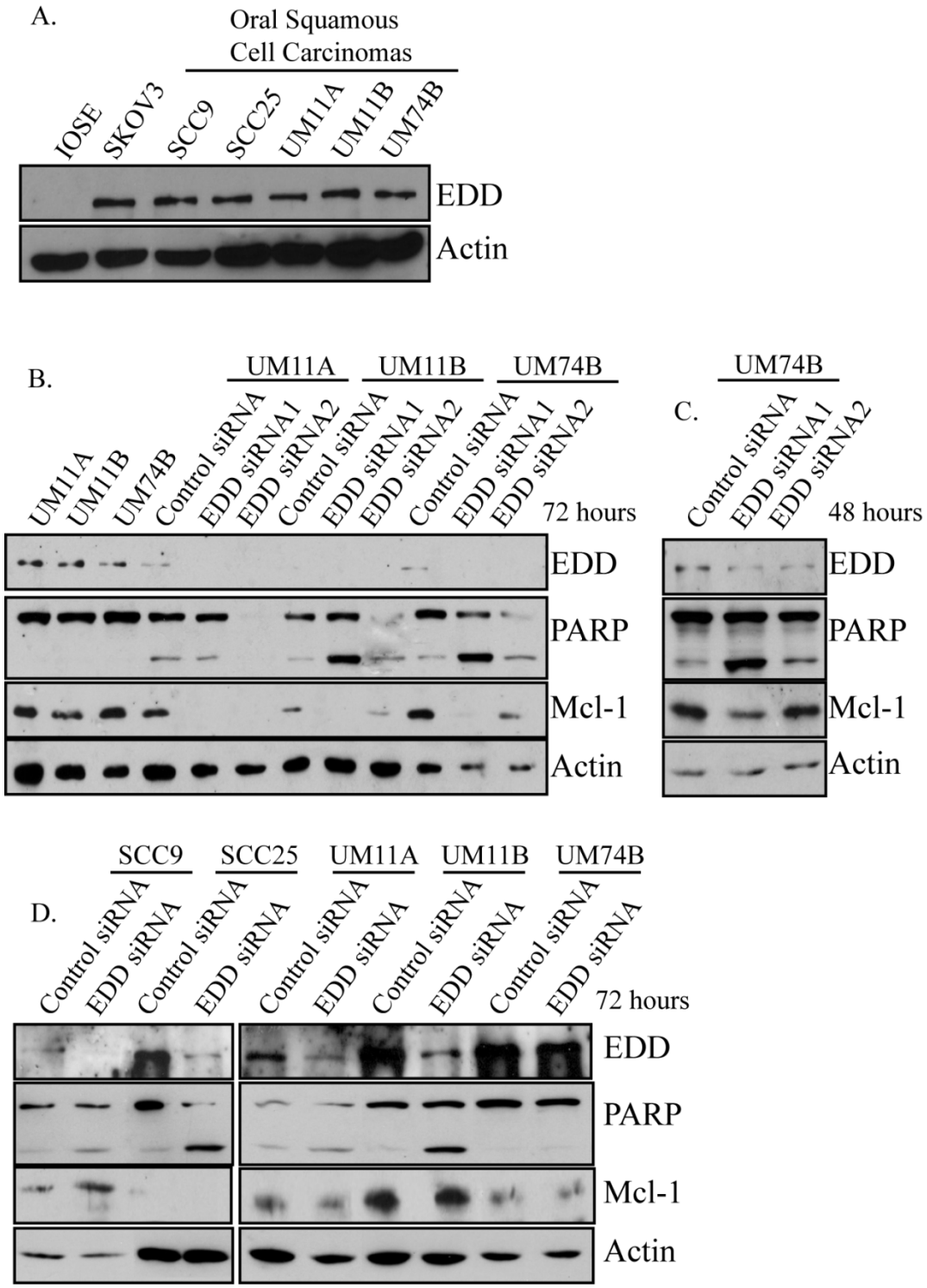

A.
Oral Squamous Cell Carcinomas
IOSE
SKOV3
SCC9
SCC25
UM11A
UM11B
UM74B
EDD
Actin
B.
UM11A
UM11B
UM74B
Control siRNA
EDD siRNA1
EDD siRNA2
72 hours
EDD
PARP
Mcl-1
Actin
C.
UM74B
Control siRNA
EDD siRNA1
EDD siRNA2
48 hours
EDD
PARP
Mcl-1
Actin
D.
SCC9
SCC25
UM11A
UM11B
UM74B
Control siRNA
EDD siRNA
72 hours
EDD
PARP
Mcl-1
Actin

Figure 4.1. SiRNA mediated EDD knockdown causes apoptosis in some oral squamous cell carcinoma cell lines. (A) Cell lysates from a benign ovarian cell line (IOSE), ovarian cancer cell line (SKOV3), and five different oral squamous cell carcinomas were immunoblotted to detect EDD protein expression. (B) UM11A, UM11B, and UM74B cell lines were transfected with control siRNA or two different siRNAs targeting EDD for 72 hours. Cell lysates were collected and blotted for EDD to detect knockdown, PARP to detect apoptosis indicated by the presence of a lower, cleaved band of PARP, the anti-apoptotic protein Mcl-1, and Actin for a loading control. (C) UM74B cells were transfected with control siRNA, EDD siRNA 1, or EDD siRNA 2 for 48 hours. Cell lysates were immunoblotted for EDD, PARP to detect apoptosis, Mcl-1, and Actin. (D) SCC9, SCC25, UM11A, UM11B, and UM74B cells were transfected with control or EDD siRNA 1 for 72 hours and immunoblotted for EDD, PARP, Mcl-1, and Actin as described above.

Transient knockdown of EDD with siRNA increases cisplatin sensitivity in some oral squamous cell carcinoma cell lines

Based on my research (Bradley, Zheng et al. 2014) and that of O'Brien et al. (O'Brien, Davies et al. 2008) in ovarian cancer, which demonstrates that EDD knockdown can increase cisplatin sensitivity, I sought to determine if the same was true in oral squamous cell carcinoma. Five different oral squamous cell carcinoma cell lines (SCC9, SCC25, UM11A, UM11B, and UM74B cells) were transfected with EDD siRNA 1 for 24 hours to knockdown EDD protein expression (Figure 4.2). Then cells were also treated with a 10 μM dose of cisplatin for an additional 24 hours. An increase in cell death, and therefore an increased sensitivity to cisplatin treatment, was determined by the relative amount of PARP cleavage in comparison to EDD siRNA only treated cells or control siRNA plus cisplatin treatment. In cell lines that demonstrated both a good knockdown of EDD and were sensitive to EDD knockdown induced apoptosis, such as SCC9, SCC25, and UM11B cells, there was also an increase in cisplatin sensitivity. In SCC9 and SCC25 cells, this is demonstrated by both a decrease in total uncleaved PARP and a slight increase in cleaved PARP. While these cell lines are sensitive to both EDD knockdown induced apoptosis and cisplatin treatment, the combination of the two demonstrates a further increase in cell death.

In UM11B cells, which are again sensitive to both apoptosis from EDD knockdown and cisplatin, the combination of the two treatments increases apoptosis as evidenced by an increase in cleaved PARP. In UM11A cells, as

shown in Figure 4.1B & C, these cells are not sensitive to EDD siRNA or cisplatin, but the combination of the two treatments does increase apoptosis indicating that EDD knockdown may sensitize these cells to cisplatin, whereas they were not sensitive previously. In this experiment, the only cell line which definitely does not show increased cisplatin sensitivity after EDD knockdown is UM74B, but this is due to the fact that there was not a good knockdown of EDD after transfection with siRNA. More experiments are needed in this cell line to determine if there is an effect of EDD knockdown to increase cisplatin sensitivity. Overall, most of the oral squamous cell carcinoma cell lines demonstrated increased cisplatin sensitivity as a result of EDD knockdown, including one cell line which was not sensitive to EDD knockdown induced apoptosis.

Figure 4.2.

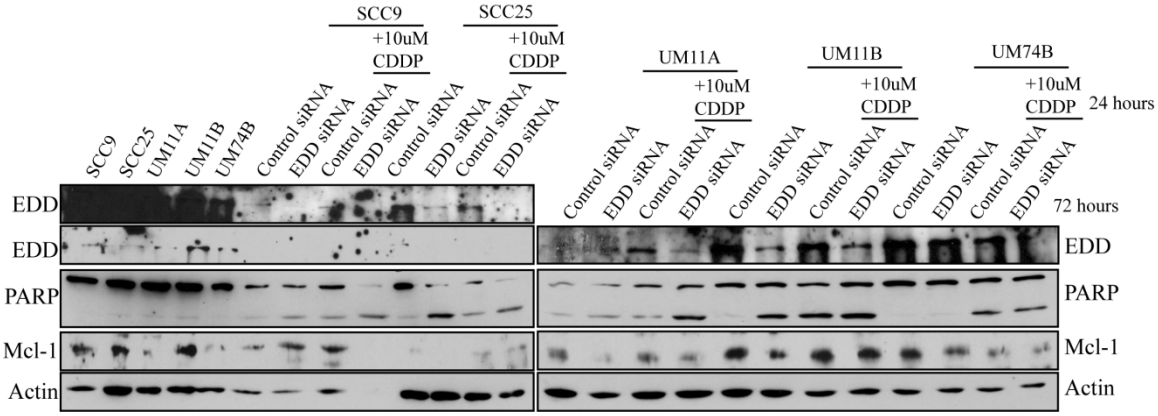


Figure 4.2. Transient knockdown of EDD may increase cisplatin sensitivity in some oral squamous cell carcinoma cell lines. SCC9, SCC25, UM11A, UM11B, and UM74B cell lines were transfected with control siRNA or EDD siRNA1 for 48 hours and then treated with 10 μM cisplatin for an additional 24 hours. Cell lysates were immunoblotted for EDD to show protein knockdown, PARP to indicate apoptosis by the cleavage of PARP, the anti-apoptotic protein Mcl-1, and actin for a loading control.

Creation of EDD stable knockdown cell lines

To further test if EDD knockdown increases cisplatin sensitivity, it is essential to separate the functions of EDD in the regulation of cellular survival from that in cisplatin resistance. A doxycycline inducible shRNA pTRIPZ plasmid was used which encodes either: control shRNA, EDD shRNA 1, EDD shRNA 2, or EDD shRNA 3. This plasmid also encodes an RFP (red fluorescent protein) expression gene for ease of determining which cells express shRNA. Once a stable population that expresses the shRNA plasmid is selected for with puromycin, then doxycycline can be used to turn on the shRNA expression to knockdown the EDD mRNA and protein expression. This approach also addresses whether EDD knockdown with shRNA causes apoptosis in these cell lines (UM22B and UM74B) similar to EDD knockdown with siRNA. After the addition of doxycycline for at least seven days, RFP expression is visible in a high percentage of the cells (about 90% in UM74B and about 60% in UM22B cells). EDD shRNA expression does not appear to cause cell death (results not shown). Figure 4.3A demonstrates the effectiveness of EDD protein expression knockdown after the induction of the EDD shRNA in UM22B cells and Figure 4.3B displays this in UM74B cells. Based on these results, it seems that EDD shRNA 1 and EDD shRNA 3 are the most effective in UM74B and EDD shRNA 3 causes some knockdown in UM22B cells.

In order to obtain a pure population of cells that express the EDD shRNA plasmid (as evidenced by RFP expression), FACS was used to sort cells for the top 25% of each stable cell lines that express the highest level of RFP. The

immunoblot for EDD expression in these stable cell lines is shown in Figure 4.3C, where UM22B cells do not have adequate knockdown of EDD, but UM74B cells expressing EDD shRNA 3 have an almost complete loss of EDD protein. These UM74B cells were tested for cisplatin sensitivity by an MTS assay to evaluate if stable knockdown of EDD increases cisplatin sensitivity. Figure 4.3D indicates that UM74B EDD shRNA 3 cells are not more sensitive to cisplatin treatment over 72 hours than UM74B scrambled shRNA stable cells. Since an MTS assay measures the relative amount of living cells by detecting mitochondrial activity, this assay may not be the most appropriate way to measure cisplatin sensitivity. Cisplatin sensitivity is also measured in these cells by a colony formation assay.

Figure 4.3.

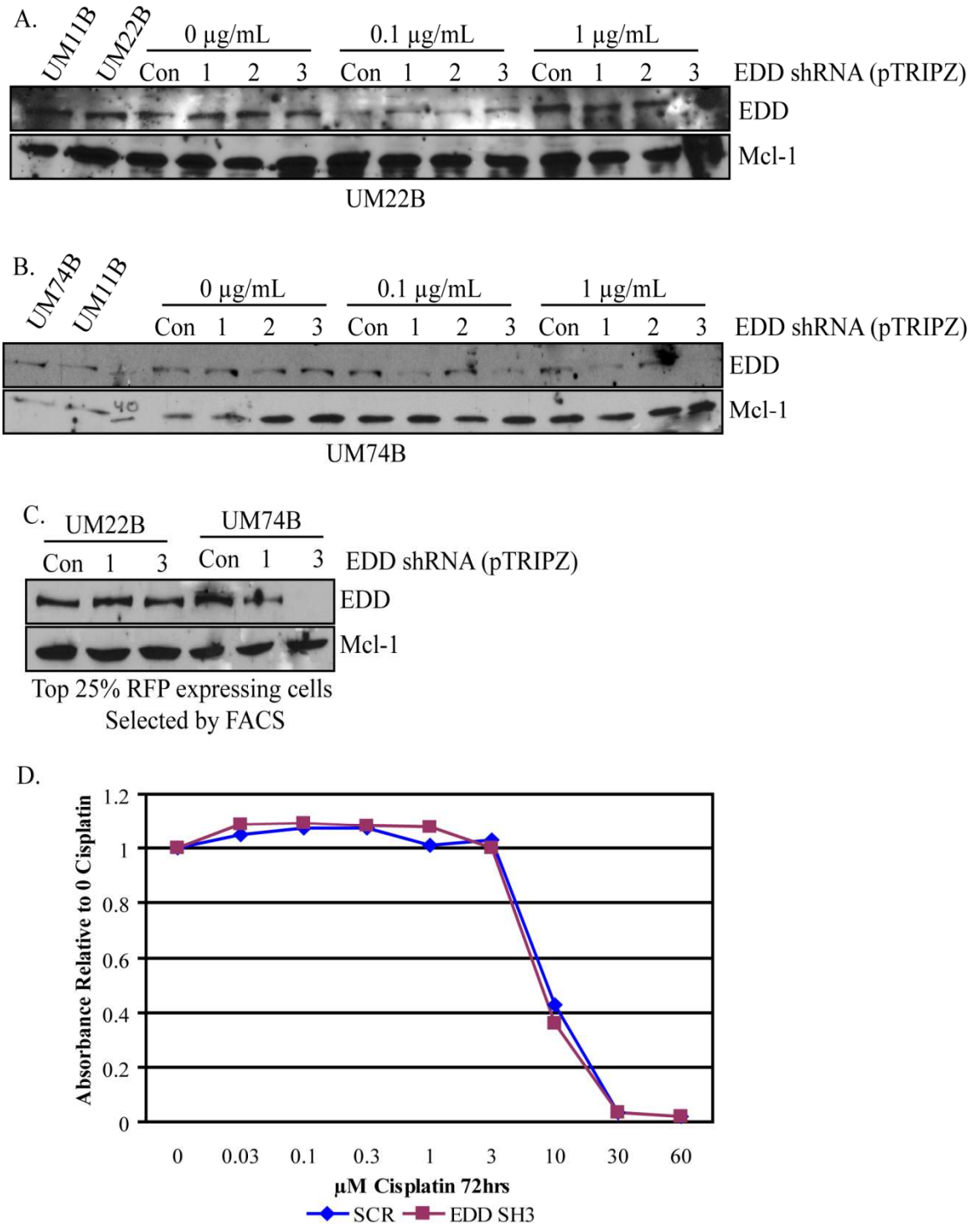

A.
UM11B
UM22B
0 µg/mL
0.1 µg/mL
1 µg/mL
Con 1 2 3 Con 1 2 3 Con 1 2 3
EDD shRNA (pTRIPZ)
EDD
Mcl-1
UM22B
B.
UM74B
UM11B
0 µg/mL
0.1 µg/mL
1 µg/mL
Con 1 2 3 Con 1 2 3 Con 1 2 3
EDD shRNA (pTRIPZ)
EDD
Mcl-1
UM74B
C.
UM22B
UM74B
Con 1 3 Con 1 3
EDD shRNA (pTRIPZ)
EDD
Mcl-1
Top 25% RFP expressing cells
Selected by FACS
D.
Absorbance Relative to 0 Cisplatin
1.2
1
0.8
0.6
0.4
0.2
0
0 0.03 0.1 0.3 1 3 10 30 60
µM Cisplatin 72hrs
SCR
EDD SH3

Figure 4.3. Inducible stable EDD knockdown in oral squamous cell carcinomas. (A) UM22B cells were transduced with a lentivirus containing the pTRIPZ plasmid which includes either a control shRNA or one of three different EDD shRNAs. After selection of a stable population containing the shRNA with puromycin selection, shRNA expression in these cells was induced by treatment with doxycycline for at least seven days. Cells treated with different doses of doxycycline (0, 0.1, or 1 µg/mL) were lysed and immunoblotted for EDD to detect knockdown and the anti-apoptotic protein Mcl-1. (B) As in (A), UM74B were transduced with control or EDD shRNA and the effectiveness of the knockdown is demonstrated in this immunoblot. (C) UM22B and UM74B cells from (A) and (B) treated with 1 µg/mL doxycycline for at least one week were sent for FACS for RFP expression which is encoded on the pTRIPZ plasmid. The top 25% of cells expressing the highest amounts of RFP were selected and cultured. These cells were lysed and immunoblotted for EDD to detect knockdown and the anti-apoptotic protein Mcl-1. (D) UM74B cells from (C) expressing either the control shRNA or the EDD shRNA 3 were tested for cisplatin sensitivity by an MTS assay over 72 hours. This is a graphical representation of one experiment with samples plated in quadruplicate. Absorbance is normalized to the average absorbance of untreated cells in each stable cell line.

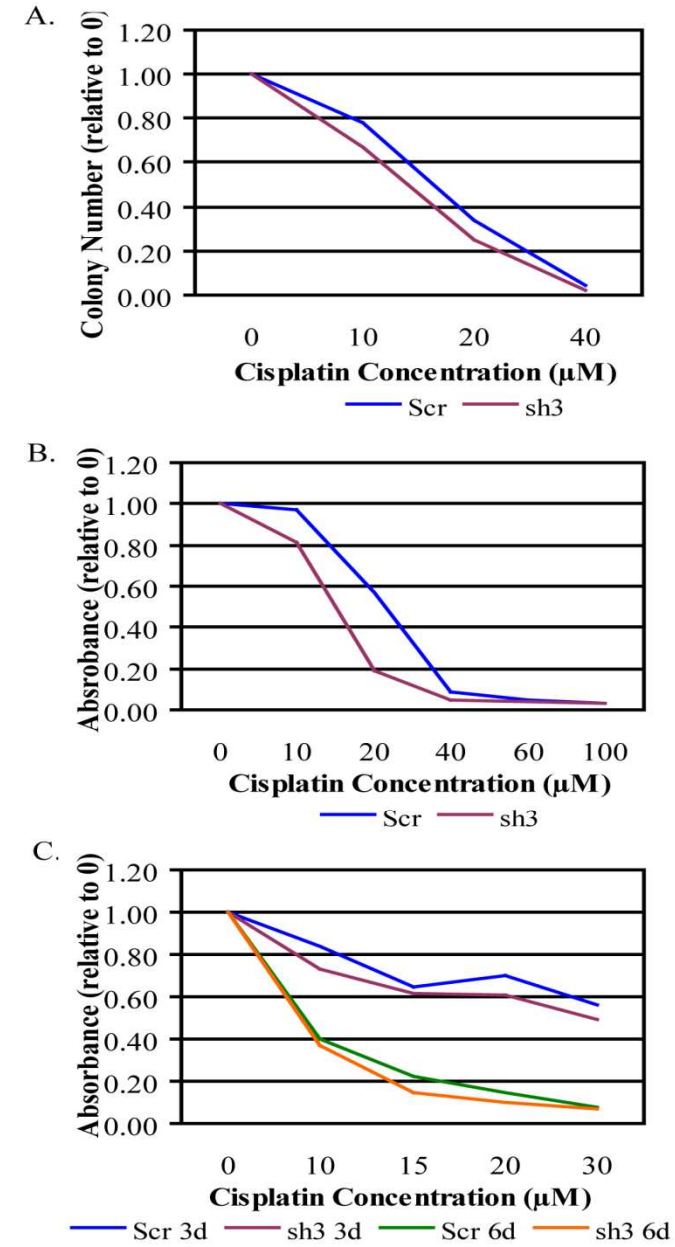
EDD stable knockdown reduces colony size in UM74B cells

Based on results from Figure 4.3D, which indicated that an MTS assay may not be the most appropriate way to measure cisplatin sensitivity in the oral squamous cell carcinoma cell lines, colony formation assays were used to test the EDD stable knockdown cells for relative cisplatin sensitivity. Colony formation assays allow for the analysis of the DNA damage response pathway since these cells are treated with cisplatin for 2 hours and allowed to repair their DNA over several days rather than 72 hours of continuous cisplatin treatment as in an MTS assay. UM74B cells, scrambled shRNA or EDD shRNA 3 overexpressing, were treated with different concentrations of the chemotherapeutic drug cisplatin (0, 10, 20, 40 μM) for 2 hours and then allowed to recover for 3 days. After 3 days, cells were collected and the amount of colonies remaining on the plate was counted (Figure 4.4A). EDD stable knockdown cells (sh3) had slightly fewer colonies (approximately 27% less at 20 μM) than control scrambled knockdown cells (Figure 4.4A). This is similar to results published by O'Brien et al in A2780-cp70 cells treated with EDD siRNA and 20 μM cisplatin (O'Brien, Davies et al. 2008).

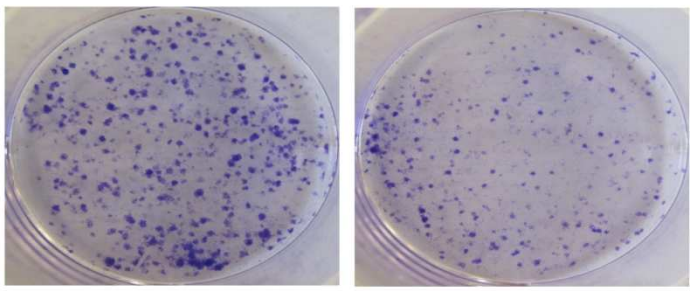
The colonies from the EDD stable knockdown cells appeared to be much smaller than those in the scrambled control cell lines when treated with cisplatin (Figure 4.4D). To determine the relative amount of cells on the plate, as determined by the total amount of crystal violet staining on the colonies, the absorbance of solubilized crystal violet was measured. EDD stable knockdown cells had 67% less (at 20 μM) crystal violet staining, and therefore theoretically

fewer cells, when treated with cisplatin than the scrambled control cells (Figure 4.4B). This difference is also evident over six days of recovery time after cisplatin treatment as well (Figure 4.4C). Based on these results, it appears that EDD stable knockdown in UM74B cells slows cellular proliferation when cells are exposed to cisplatin treatment.

Figure 4.4.



D.



Scrambled
20µM Cisplatin

EDD sh3
20µM Cisplatin

Figure 4.4. UM74B stable EDD knockdown cells demonstrate reduced colony size in cisplatin sensitivity colony formation assays. (A) UM74B scrambled control shRNA expressing cells and UM74B EDD shRNA3 expressing cells were plated sparsely, treated for 2 hours with different concentrations of cisplatin, and colonies were allowed to grow for 3 days before being collected and stained with crystal violet. The number of colonies was counted and the results from one experiment are depicted in this graph. The number of colonies in untreated cells is set to 1 and other conditions are normalized to this number of colonies in the control. (B) As in (A), after colonies were counted, the crystal violet staining the colonies was solubilized with 2% SDS and the absorbance was measured at 550 nm. Solubilized crystal violet allows for relative quantitation of total crystal violet staining taking into account both colony number and colony size. This graph is a representation of one experiment and absorbance is normalized to the untreated control cells in each cell line. (C) As in (B), colonies from each stable cell line were allowed to grow for 3 or 6 days after 2 hours of cisplatin treatment. Absorbance of solubilized crystal violet staining is normalized to untreated cells. This graph is a representation of a single experiment. (D) Scrambled shRNA expressing cells and EDD shRNA3 expressing cells were collected 3 days after 2 hours of 20 μM cisplatin treatment as in (A). These pictures are used to demonstrate the relative number of colonies and size of colonies on each plate that were used in (A) and (B).

EDD stable knockdown does not affect proliferation across oral squamous cell carcinoma cell lines

To resolve whether EDD stable knockdown has an effect on cellular proliferation rather than on cisplatin sensitivity, I performed colony formation assays over time in untreated cells. In order to have another cell line to compare the UM74B EDD stable knockdown cell line, UM22B cells were transduced with an EDD shRNA retroviral vector. The UM22B EDD stable knockdown cell line expressing the UTR (untranslated region) targeted shRNA showed the greatest knockdown of EDD protein expression as compared to the scrambled control (Figure 4.5A). A proliferation assay was used to measure colony growth over 7 days daily in UM22B and UM74B scrambled control shRNA and EDD shRNA expressing cells. There was not a significant difference in cellular proliferation in the UM22B scrambled and EDD knockdown cell lines over time, but UM74B EDD stable knockdown cells did grow 31% slower than their scrambled control counterpart at day 7 (Figure 4.5B).

In order to verify that the effect seen in the UM74B cells is due to EDD knockdown and not a coincidence of the stable cell line population simply having a slower rate of proliferation, I performed a proliferation assay with the UM74B cells scrambled and EDD knockdown with and without doxycycline. Without doxycycline to activate expression of the shRNA in the cells, the shRNA will not be expressed and the cells will return to a basal expression of EDD protein. Figure 4.5C illustrates that the UM74B cells without doxycycline do not grow significantly slower than the populations treated with doxycycline and therefore

expressing shRNA (either scrambled or EDD shRNA 3). Comparison of UM74B scrambled cells with doxycycline to EDD shRNA 3 cells with doxycycline in this experiment shown in Figure 4.5C indicates that EDD stable knockdown cells do not have a reduced rate of proliferation in this experiment even though the EDD knockdown is still present (results not shown). Due to this inconsistency, more experiments are needed to finally determine if EDD stable knockdown does indeed reduce proliferation in the UM74B cells. There is not an effect on the proliferation in the UM22B cells demonstrating that an effect of EDD knockdown may only be relevant to certain cell lines.

Figure 4.5

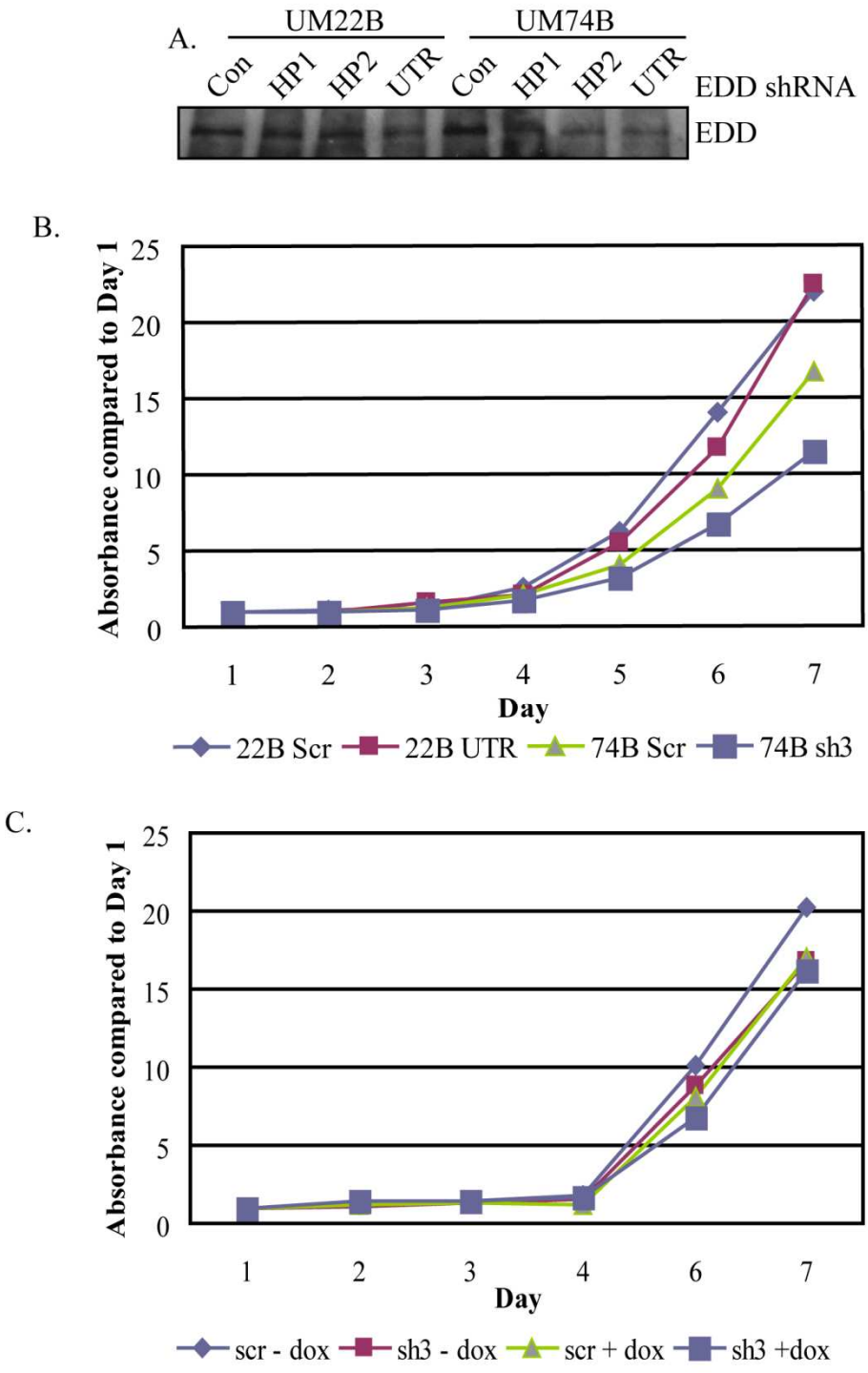

A.
UM22B
UM74B
Con
HP1
HP2
UTR
Con
HP1
HP2
UTR
EDD shRNA
EDD
B.
Absorbance compared to Day 1
25
20
15
10
5
0
1
2
3
4
5
6
7
Day
22B Scr
22B UTR
74B Scr
74B sh3
C.
Absorbance compared to Day 1
25
20
15
10
5
0
1
2
3
4
6
7
Day
scr - dox
sh3 - dox
scr + dox
sh3 +dox

Figure 4.5. UM74B EDD stable knockdown cells show a reduction in proliferation. (A) Non-inducible shRNAs, either control or shRNA targeting EDD (HP2, HP2, and UTR), were transduced into UM22B and UM74B cells, then populations were selected with puromycin. Cell lysates were collected from these populations and immunoblotted for EDD to detect protein knockdown. These same shRNAs were also used in ovarian cancer cells as described in Chapter 3. (B) UM22B scrambled control shRNA and UM22B EDD shRNA UTR cells from (A) and UM74B scrambled control shRNA and UM74B EDD shRNA 3 from (Figure 4.3C) were used in a colony formation assay. Cells were plated sparsely in doxycycline and colonies after a certain number of days from 1-7 were collected and stained with crystal violet. Crystal violet staining from each experimental plate of cells was solubilized with 2% SDS and absorbance was measured at 550nm. Absorbance of each experimental condition was normalized to cells from day 1. (C) UM74B scrambled and EDD shRNA 3 expressing cells either treated with doxycycline for 7 days prior to the experiment and during the experiment were compared to the same cells that were not treated with doxycycline. Cells were collected over time as in (B) and absorbance of solubilized crystal violet in each experimental plate was normalized to cells at day 1 from each treatment condition.

EDD stable knockdown sensitizes UM22B cells to cisplatin

Similar to the colony formation assay performed in Figure 4.4, UM22B stable populations from Figure 4.5A (scrambled shRNA and EDD UTR targeting shRNA expressing) were treated with cisplatin for 2 hours and colonies were allowed to recover for three days. Cells were treated with different concentrations of cisplatin (0, 10, 15, 20, 30, or 40 µM). Results in Figure 4.6A show that at certain concentrations of cisplatin, UM22B EDD stable knockdown cells are more sensitive to cisplatin than their scrambled control shRNA counterparts. At a 20 µM dose of cisplatin, EDD knockdown cells are 37% more sensitive to cisplatin than the scrambled control cell line. This is consistent with results from O'Brien et al. which demonstrated a similar effect in A2780 cisplatin resistant cells treated with either scrambled siRNA or EDD siRNA and treated with a 20 µM dose of cisplatin (approximately 40% increase in sensitivity) (O'Brien, Davies et al. 2008). Similar to results in Figure 4.5B, Figure 4.6B illustrates that the colony size in scrambled and EDD shRNA expressing stable cell lines is relatively the same, indicating that EDD knockdown is not affecting the rate of proliferation in these cells. A visual representation of the colonies counted in Figure 4.6A is displayed in Figure 4.6C (UM22B scrambled shRNA expressing cells) and Figure 4.6D (UM22B EDD UTR targeting shRNA expressing cells). Although there is a qualitative difference in the number of colonies present in cells treated with 15 µM, 20 µM, and 30 µM doses of cisplatin indicating that EDD stable knockdown increases cisplatin sensitivity in UM22B cells treated with these certain doses of cisplatin, otherwise the effect is not significant. These results demonstrate a

trend towards EDD knockdown fostering cisplatin sensitivity, but more experiments are required in order to draw a statistically significant conclusion.

Figure 4.6

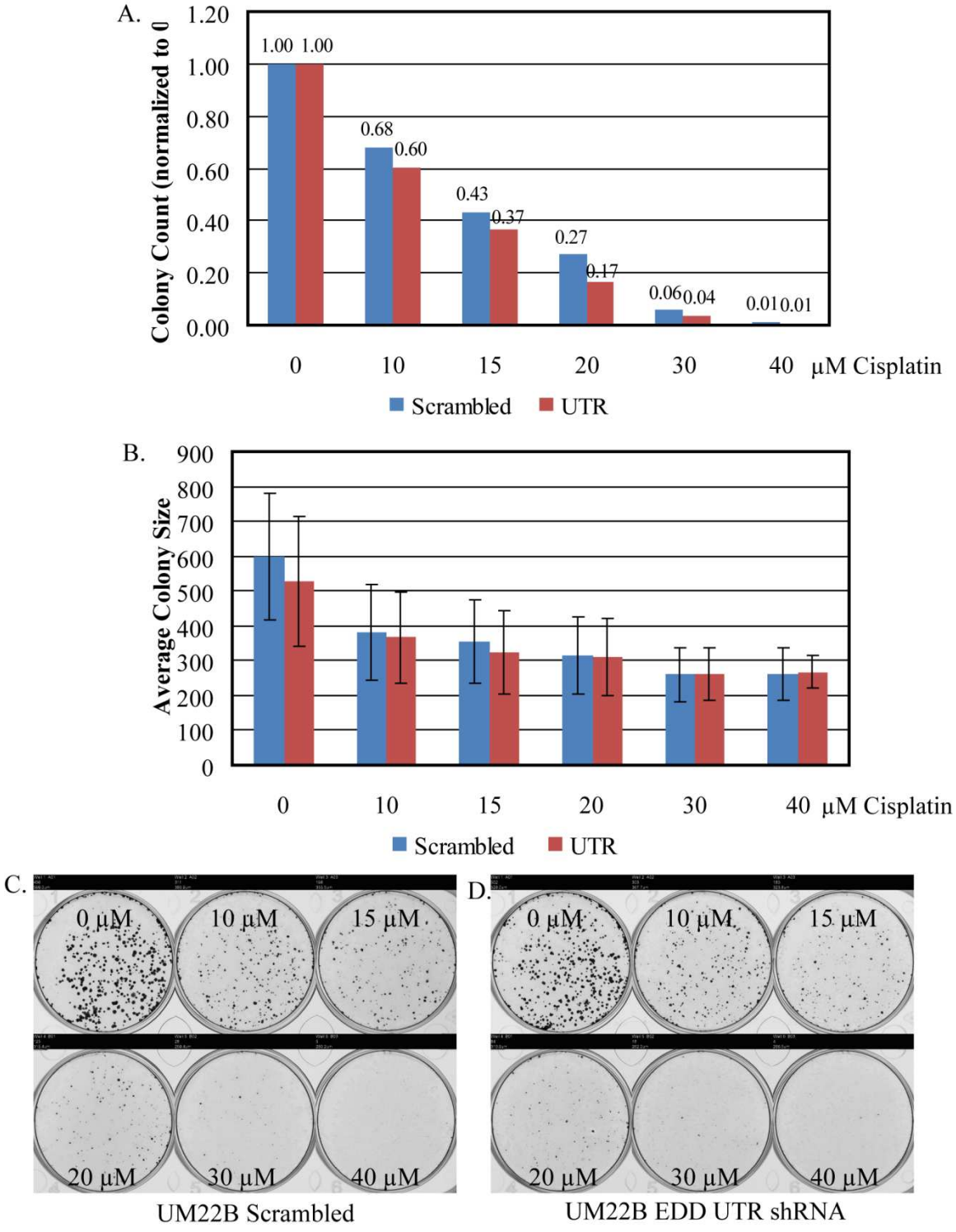

A.
Colony Count (normalized to 0
1.20
1.00
0.80
0.60
0.40
0.20
0.00
1.00 1.00
0.68
0.60
0.43
0.37
0.27
0.17
0.06 0.04
0.01 0.01
0
10
15
20
30
40 µM Cisplatin
Scrambled
UTR
B.
Average Colony Size
900
800
700
600
500
400
300
200
100
0
0
10
15
20
30
40 µM Cisplatin
Scrambled
UTR
C.
0 µM
10 µM
15 µM
20 µM
30 µM
40 µM
UM22B Scrambled
D.
0 µM
10 µM
15 µM
20 µM
30 µM
40 µM
UM22B EDD UTR shRNA

Figure 4.6. UM22B stable EDD knockdown cell lines demonstrate increased cisplatin sensitivity. (A) UM22B scrambled shRNA and EDD UTR shRNA cell lines from Figure 4.5A were tested in a colony formation assay to detect changes in cisplatin sensitivity. Cells were plated sparsely and treated with increasing concentrations of cisplatin (0, 10, 15, 20, 30, or 40 μM) for 2 hours and then colonies were allowed to grow for 3 days. Colonies were counted and normalized to the amount of colonies on the untreated plates. (B) Colonies from (A) were analyzed for average colony size to determine any differences in the rate of proliferation in the two different stable cell lines. Pictures of the colonies analyzed in (A) and (B) are depicted in (C) UM22B scrambled control shRNA overexpressers and (D) UM22B EDD UTR shRNA overexpressers.

Discussion

There are certain parallels between ovarian cancer and oral squamous cell carcinoma cell lines in regards to EDD. As in ovarian cancer, EDD knockdown in some oral squamous cell carcinoma cell lines promotes apoptosis, although unlike ovarian cancer, this does not appear to be through regulation of Mcl-1. EDD knockdown, either transiently or stably, also increases cisplatin sensitivity in some oral squamous cell carcinoma cell lines. The *edd* gene is overamplified in 50% of oral squamous cell carcinoma of the tongue and in 47% of all types of ovarian cancer (Clancy, Henderson et al. 2003). A commonality between these two completely different types of cancer that led me to believe that EDD may be involved in their cellular signaling process is that in addition to the amplification of *edd*, both are commonly treated with the DNA-damaging chemotherapeutic cisplatin and both cancers often display resistance to this drug. As I have previously shown in ovarian cancer, EDD mediates cellular survival through the transcriptional regulation of the anti-apoptotic protein Mcl-1 and promotes cisplatin resistance through EDD's ubiquitin ligase function (Bradley, Zheng et al. 2014). Before beginning this project exploring the relationship between EDD and oral squamous cell carcinoma, there was no connection between this ubiquitin ligase and this cancer other than its gene amplification.

Presently, the data provided in this chapter demonstrate that the EDD protein is highly expressed in oral squamous cell carcinoma cell lines and loss of EDD, in addition to promoting apoptosis in some of these cell lines, can also

cause cisplatin sensitivity. EDD knockdown induced apoptosis did not appear to be dependent on regulation of the anti-apoptotic protein Mcl-1. Mcl-1 protein expression levels were not consistently decreased in response to EDD knockdown as was seen in ovarian cancer cell lines. This indicates that some of these cell lines may not be dependent on Mcl-1 or EDD for cellular survival. As expected, oral squamous cell carcinoma cell lines use different cellular signaling pathways to regulate survival than ovarian cancer and this can also vary between cell lines. Bcl-2 may play a more important role in regulating survival in these cell lines since it has been linked to tumor progression in oral squamous cell carcinoma (Chen, Kayano et al. 2000).

Cell lines (SCC9, SCC25, UM11B) which experienced effective knockdown of EDD in response to siRNA transfection were also more sensitive to cisplatin. More significantly, one cell line (UM11A), which was consistently not susceptible to EDD knockdown induced apoptosis or cisplatin treatment, was sensitive to the combination of the two. This indicates that transient knockdown of EDD may not be regulating survival in this particular cell line, but it can control cisplatin sensitivity. In order to separate EDD's role in maintaining cellular survival and cisplatin resistance, EDD stable knockdown cell lines were created. In these cell lines, created with an inducible EDD shRNA, induction of EDD knockdown did not cause apoptosis as with siRNA mediated knockdown, further complicating our understanding of signaling pathways in these cells and EDD's potential role to promote survival. These stable cell lines may have adopted alternative signaling pathways to allow for survival without the presence of EDD.

As seen previously, different cell lines can have vastly different responses in regards to EDD's regulation of cisplatin resistance. In UM74B cells, there was no clear evidence of EDD stable knockdown cells experiencing increased apoptosis when treated with cisplatin. In this cell line, it appears that loss of EDD may reduce proliferation when cells are treated with cisplatin. UM74B EDD stable knockdown cells also demonstrated inconsistent results in proliferation assays without cisplatin treatment, indicating that these cells may exhibit defects in proliferation due to EDD knockdown. This is explicable because EDD has been previously shown in multiple publications to regulate the cell cycle and response to DNA damage (Henderson, Russell et al. 2002, Gupta, Chakrobarty et al. 2006, Henderson, Munoz et al. 2006, Munoz, Saunders et al. 2007, Ling and Lin 2011, Smits 2012). Additional experiments need to be completed in UM74B EDD stable knockdown cells to examine their ability to respond to other DNA damaging agents and the mechanism behind this response. Alternatively in UM22B cells with a stable knockdown of EDD, there was no effect on the proliferation of these cells when treated with cisplatin. Results from colony formation assays in UM22B cells indicated that EDD loss in these cells increases cisplatin sensitivity at certain concentrations of cisplatin (20 μM and 30 μM), which is comparable to results shown in O'Brien's research (O'Brien, Davies et al. 2008). The results described above are summarized in Table 4.1.

Based on these results, it is difficult to draw a direct connection between EDD and cellular survival or cisplatin resistance, but further experimentation with other oral squamous cell carcinoma cell lines may lead to more conclusions. The

cell lines used in these experiments generated considerably different results, so further exploration of other cell lines may prove to be more beneficial than trying to focus on a few distinct cell lines. Alternatively, these cell lines (UM74B and UM22B) could be used for more mechanistic studies to determine the cellular signaling pathways utilized by each cell line to control responses to DNA damage, survival, and proliferation. This would allow the delineation of DNA damage response pathways and cell cycle control pathways in UM74B cells which demonstrate an alternative rate of proliferation. Ubiquitin ligase function of EDD could be further explored in UM22B cells, as these cells exhibit increased cisplatin sensitivity due to the loss of EDD, but only at certain concentrations of cisplatin. This suggests that a balance may be occurring in these cells between initial response to DNA damage and a pathway regulated by EDD's ubiquitination of another protein at moderate levels of DNA damage when repair is not effective.

Table 4.1.

	EDD siRNA			EDD shRNA		
Cell Line	**Effective siRNA Knock-down**	**Apoptosis**	**Increased Cisplatin Sensitivity**	**Effective shRNA Knock-down**	**Apoptosis**	**Increased Cisplatin Sensitivity**
UM11A	Yes	No	Yes	Not tested	Not tested	Not tested
UM11B	Yes	Yes	Yes	Not tested	Not tested	Not tested
UM74B	Yes	Yes	Inefficient knockdown	Yes	No	Reduced proliferation
Scc9	Yes	Yes	Yes	Not tested	Not tested	Not tested
Scc25	Yes	Yes	Yes	Not tested	Not tested	Not tested
UM22B	Not tested	Not tested	Not tested	Yes	No	Increased: 14% at 15 µM 37% at 20 µM 33% at 30 µM

Table 4.1. Effects of transient and stable EDD knockdown on apoptosis and cisplatin sensitivity. Summary of results depicted in Figures 4.1- 4.6 regarding EDD transient knockdown with siRNA and stable knockdown with shRNA.

Chapter 5

Conclusion

Conclusion

The purpose of this dissertation is to examine the role of the E3 ubiquitin ligase EDD in modulating cellular survival and cisplatin resistance in both ovarian cancer and oral squamous cell carcinoma. My experimental approach is as follows: 1) establish if EDD knockdown with siRNA induces apoptosis; 2) examine alterations in pro-apoptotic proteins and anti-apoptotic proteins in response to EDD knockdown induced apoptosis; 3) determine the mechanism by which EDD alters expression of apoptosis regulatory proteins; 4) determine if EDD knockdown transiently or stably promotes cisplatin sensitivity *in vitro* and *in vivo*; 5) establish if EDD overexpression is sufficient to promote cisplatin resistance; 6) resolve if EDD knockdown transiently or stably induces apoptosis and increases cisplatin sensitivity in oral squamous cell carcinoma.

EDD knockdown induces apoptosis through transcriptional regulation of Mcl-1

Since EDD is overexpressed in ovarian cancer and oral squamous cell carcinoma, siRNA was used to knockdown EDD in order to determine what cellular processes required EDD. When EDD is knocked down, cells undergo apoptosis, which is accompanied by a consistent loss in the anti-apoptotic protein Mcl-1 in ovarian cancer cell lines. EDD knockdown with siRNA in SCC25, UM11B, and UM74B oral squamous cell carcinoma cell lines causes apoptosis, without a consistent change in Mcl-1. This indicates the variety of cellular

signaling pathways employed by these cell lines as compared to other oral squamous cell carcinoma cell lines tested and the signaling pathways in ovarian cancer cell lines.

In ovarian cancer, even when apoptosis is inhibited before EDD knockdown can cause cleavage of caspases, Mcl-1 protein expression is still decreased. These cells apparently have an equal requirement for EDD and Mcl-1 for cellular survival as a loss of either results in 23% apoptosis in ES-2 cells and 41% in A2780-ip2 cells. Overexpression of Mcl-1 is sufficient to protect cells from EDD knockdown induced apoptosis, even though EDD knockdown still results in a loss of endogenous Mcl-1. Due to the alternative regulation of endogenous versus exogenous Mcl-1, this led to the deduction that EDD is regulating Mcl-1 at its promoter, since the exogenous Mcl-1 is driven by a CMV promoter instead of the endogenous promoter. This assumption was supported by real time PCR in which EDD knockdown causes a 1.87 fold decrease in Mcl-1 mRNA. Furthermore, both wild-type EDD and an EDD mutant which lacks ubiquitin ligase activity were both found to activate transcription from either a direct or indirect action on the *mcl-1* promoter as found in Mcl-1 promoter driven luciferase assays. These results illustrate that EDD enhances cellular survival in ovarian cancer cell lines through transcriptional regulation of *mcl-1* at its promoter. Furthermore, this is independent of EDD's ubiquitin ligase activity.

EDD knockdown sensitizes cells to cisplatin

In addition to being overexpressed in ovarian and oral squamous cell carcinoma of the tongue, EDD is also associated with a 2-fold increased risk of

disease recurrence and death in patients that initially responded to chemotherapy treatment (Clancy, Henderson et al. 2003, O'Brien, Davies et al. 2008). In addition, the amplification of the chromosomal region surrounding EDD is associated with cisplatin resistance (Wasenius, Jekunen et al. 1997, Callaghan, Russell et al. 1998). To determine the significance of EDD in regulating cisplatin resistance in ovarian cancer and oral squamous cell carcinoma of the tongue, EDD was knocked down transiently with siRNA or stably with shRNA. In the ovarian cancer cell line ES-2, knockdown of EDD with siRNA causes apoptosis alone, but when combined with a low dose of cisplatin treatment, this considerably enhances apoptosis in this cell line. In another ovarian cancer cell A2780-ip2, knockdown of EDD with siRNA caused such a substantial amount of apoptosis that it masked any additional effect from cisplatin treatment. To separate EDD's functions regarding EDD knockdown induced apoptosis and increased cisplatin sensitivity, EDD stable knockdown cell lines were created with EDD shRNA. These cell lines confirmed results that EDD knockdown increases cisplatin sensitivity across three ovarian cancer cell lines. EDD stable knockdown cell lines did not show a decrease in the anti-apoptotic protein Mcl-1 indicating that these two functions of EDD are separate.

Transient knockdown of EDD with siRNA increases cisplatin sensitivity in some oral squamous cell carcinoma cell lines, such as SCC9, SCC25, UM11B, and UM11A. Unexpectedly, UM11A cells, which are typically not sensitive to EDD knockdown induced apoptosis, experienced apoptosis when treated with both EDD siRNA and cisplatin. This again supports the display of variety across

the oral squamous cell carcinoma cell lines as well as confirms a role for EDD in regulating cisplatin sensitivity. This diversity also creates variability in the results observed in UM74B and UM22B cells with a stable knockdown of EDD using shRNA expression. UM74B cells appear to exhibit a slower rate of proliferation when EDD is lost in EDD shRNA stable expressing cells as compared to control shRNA expressing cells when these cells are treated with cisplatin. Inversely, UM22B cells with stable knockdown of EDD do not exhibit any alterations in proliferation with or without cisplatin treatment. These UM22B stable EDD knockdown cell lines do demonstrate a trend of increased cisplatin sensitivity due to EDD stable knockdown at certain cisplatin concentrations (20 μM and 30 μM). Thus, while more experiments are needed in the oral squamous cell carcinoma cell lines to examine various cell lines, analysis in both ovarian cancer and oral squamous cell carcinoma demonstrates that knockdown of EDD, transiently and stably, increases cisplatin sensitivity.

EDD overexpression is sufficient to promote cisplatin resistance, dependent on its ubiquitin ligase activity

In order to determine the significance of EDD overexpression in relationship to cisplatin resistance, COS-7 cells were transfected with GFP (control), wild-type EDD, or C2768A EDD mutant (ubiquitin ligase defective). After treatment with cisplatin, the percentage of those cells undergoing apoptosis was measured using a kit that enabled detection by microscopy. Cells that were transfected with wild-type EDD had significantly fewer cells undergoing apoptosis due to cisplatin treatment, indicating increased cisplatin resistance in these cells.

Cells transfected with the ubiquitin ligase defective mutant of EDD (C2768A) did not increase cisplatin resistance in these cells when compared to the GFP control transfected cells. As this mutant differs at only one amino acid (cysteine 2768 to alanine), which prevents ubiquitin transfer to a substrate of EDD, this implies that the ubiquitin ligase function of EDD is required to mediate cisplatin resistance in these cells. While these experiments were performed in a non-cancerous cell line, this indicates that EDD is sufficient when overexpressed to promote cisplatin resistance, which may be translatable to cancerous cells since EDD knockdown increases cisplatin sensitivity in these cells lines. Stable cell lines overexpressing EDD or better transfection efficiency is necessary before similar experiments could be performed in cancerous cell lines.

EDD is a potential therapeutic target in ovarian cancer

E3 ubiquitin ligases have a catalytic domain, but do not possess a defined catalytic pocket that would allow small molecule inhibitors to bind. For this reason, DOPC liposome nanoparticles which encapsulate siRNA targeting EDD were utilized by our collaborators to deliver EDD siRNA *in vivo* to athymic nude mice xenograft models of ovarian cancer. Mice were injected with ovarian cancer cell lines (ES-2 or A2780-ip2) intraperitoneally and tumors were allowed to develop for one week. Then mice were treated with control or EDD siRNA in nanoparticles with or without cisplatin treatment. After four weeks of treatment, tumors were harvested to reveal that EDD siRNA (without cisplatin) caused a decrease in tumor burden and when EDD siRNA was combined with cisplatin treatment this caused a significant reduction in total tumor burden (ES-2 –

77.9%; A2780-ip2-75.9% reduction compared to control siRNA only treated). This provides further support for the knockdown of EDD promoting apoptosis and enhancing cisplatin sensitivity by demonstrating this effect *in vivo* as a potential therapeutic strategy. The DOPC liposome nanoparticles encapsulating EDD siRNA to knockdown EDD is a more attractive therapeutic strategy because loss of EDD would decrease cellular survival while also increasing cisplatin sensitivity. If a small molecule inhibitor was used, it would likely only affect one of these two pathways (either survival or cisplatin sensitivity) since these two pathways are functionally distinct based on the function of EDD as either a transcriptional co-activator or an E3 ubiquitin ligase.

Conclusion

In conclusion, this dissertation demonstrates that EDD regulates cellular survival and cisplatin resistance in both ovarian cancer and oral squamous cell carcinoma. In ovarian cancer, EDD regulates the transcription of the anti-apoptotic protein Mcl-1 at both proximal and distal regions of its promoter. This occurs most likely by EDD acting as a transcriptional co-activator either directly or indirectly. As a result of EDD's regulation of Mcl-1, knockdown of EDD transiently with siRNA promotes apoptosis. Knockdown of EDD, transiently or stably, increases cisplatin sensitivity in ovarian cancer. Correspondingly, EDD overexpression is sufficient to increase cisplatin resistance which is dependent on its ubiquitin ligase function. These results are also confirmed by *in vivo* mouse xenograft studies which exhibit EDD knockdown with DOPC liposome nanoparticles encapsulating siRNA can sensitize ovarian tumors to cisplatin.

In oral squamous cell carcinoma cells lines, diversity among the cell lines generates different results depending on the cell line used. Transient knockdown of EDD causes apoptosis in most oral squamous cell carcinoma cell lines, but this effect does not appear to be dependent on regulation of Mcl-1. Stable knockdown of EDD causes a reduced rate of proliferation when UM74B cells are treated with cisplatin and knockdown of EDD increases cisplatin sensitivity in UM22B cells treated with certain concentrations of cisplatin.

Context in the Field

While initial therapies for both ovarian and oral squamous cell carcinomas are surgical debulking, chemotherapy often follows especially in highly aggressive cases. Resistance to chemotherapy, such as the DNA damaging agent cisplatin, is very common. The research in this dissertation concludes that the E3 ubiquitin ligase EDD is involved with mediating cisplatin resistance in these cancers. This is of significant importance because the *edd* gene is overamplified in both of these types of cancers, including breast, hepatocellular carcinoma, and metastatic melanoma (Clancy, Henderson et al. 2003, Fuja, Lin et al. 2004). Previously my mentor Scott Eblen also identified EDD as a direct substrate of ERK2, implicating EDD as a valuable protein involved in mediating cell signaling process (Henderson, Russell et al. 2002, Eblen, Kumar et al. 2003, Bethard, Zheng et al. 2011). There are numerous publications describing EDD's roles in DNA damage response, cell cycle control, and transcriptional co-activator. This dissertation research expands upon this knowledge and integrates it to further demonstrate EDD as a regulator of Mcl-1 transcription to promote cellular survival and illustrate EDD as an E3 ubiquitin ligase that is sufficient to increase cisplatin resistance. While more mechanistic studies are needed to delineate the effectors EDD is interacting with or ubiquitinating to elicit these effects, this research is critical to establishing EDD as a regulator of cell survival and drug resistance in two different cancers in which EDD is overexpressed.

EDD was first identified as an ortholog in *Drosophila melanogaster* which identified EDD as a critical regulator of proliferation and differentiation (Mansfield,

Hersperger et al. 1994). This role is supported by my findings that EDD regulates cellular survival and may control cellular proliferation in certain oral squamous cell carcinoma cell lines. Similarly, EDD's role as a transcriptional co-activator for myocardin, the vitamin D receptor, and the progesterone receptor to control progestin-induced genes independent of ubiquitin ligase function (Henderson, Russell et al. 2002, Hu, Wang et al. 2010) is supported by this research. In addition, EDD regulated the transcription of ACVRL1 to modulate angiogenesis, and this was also shown to be independent of ubiquitin ligase activity (Chen, Yang et al. 2013). My documentation of EDD as a transcriptional regulator at the Mcl-1 promoter further confirms EDD's role as a transcriptional co-activator, independent of its ubiquitin ligase activity. Further research is needed to determine which transcription factors EDD is controlling, either directly or indirectly, to affect transcription.

In patients that initially responded to chemotherapy, EDD is associated with a 2-fold increased risk of disease recurrence and death (O'Brien, Davies et al. 2008). On a genetic level, the amplification of the chromosomal region surrounding EDD is also linked to increased cisplatin resistance (Wasenius, Jekunen et al. 1997, Callaghan, Russell et al. 1998). Numerous studies have been published regarding proteins that may regulate cisplatin resistance, but most of this research has not been able to establish if their protein of interest is sufficient to induce cisplatin resistance. My research builds on the foundations of these studies establishing potential mechanisms of cisplatin resistance to include results demonstrating that EDD overexpression is sufficient to promote cisplatin

resistance, dependent on its ubiquitin ligase activity (Bradley, Zheng et al. 2014). In addition, my research also confirms that knockdown of EDD increases cisplatin sensitivity in ovarian cancer. Preliminary results in oral squamous cell carcinoma cell lines also support this conclusion to expand this knowledge across distinct types of cancers.

EDD facilitates the DNA damage response that could be occurring in these cells as a result of cisplatin treatment through the ubiquitination and designated degradation of Topoisomerase II-binding protein (TopBP1) to prevent protection of DNA damaged chromosome ends (Honda, Tojo et al. 2002). Similarly, EDD interacts with CHK2, a checkpoint kinase during DNA damage, and enables its activating phosphorylation in order to arrest the DNA damaged cells in mitosis to allow for repair (Henderson, Munoz et al. 2006). Loss of EDD disrupts cell cycle checkpoints leading to premature, unregulated mitosis and thus polyploidy (Munoz, Saunders et al. 2007). Recently EDD has also been shown to cooperate with TRIP12 to maintain RNF168 expression to aid in the prevention of chromatin ubiquitin spreading to undamaged chromosomes (Gudjonsson, Altmeyer et al. 2012, Okamoto, Bartocci et al. 2013). While EDD's regulation of these proteins was not analyzed in my experiments, my results demonstrating EDD increases cisplatin resistance further confirms that EDD has a role in regulating DNA damage response and narrows down EDD's role as that of an E3 ubiquitin ligase.

Presently, the only relationship that has been established between EDD and oral squamous cell carcinoma is the gene amplification of *edd*. Despite the

various cellular signaling pathways employed by the oral squamous cell carcinoma cell lines, my results demonstrate that EDD regulates cellular survival in a subset of these cell lines. While more experiments are needed to be able to make direct conclusions, stable knockdown of EDD seems to slow cellular proliferation due to cisplatin treatment in UM74B cells and knockdown appears to cause a trend towards increasing cisplatin sensitivity in UM22B cells. The effects seen in the UM74B cells can be explained based on previous research describing EDD's ability to regulate the cell cycle and DNA damage response (Mansfield, Hersperger et al. 1994, Henderson, Russell et al. 2002, Gupta, Chakrobarty et al. 2006, Henderson, Munoz et al. 2006, Munoz, Saunders et al. 2007, Ling and Lin 2011, Smits 2012).

Future Directions

Determine the transcriptional co-factor(s) required for EDD's regulation of *mcl-1* transcription

Published results from this dissertation identify EDD as a transcriptional co-activator for the transcription of the anti-apoptotic protein Mcl-1, which regulates cellular survival in ovarian cancer cell lines (Bradley, Zheng et al. 2014). This effect was determined to be independent of EDD's ubiquitin ligase activity. Henderson et al. has also established EDD as a transcriptional co-activator with the progesterone receptor through an interaction in the middle section of EDD. Transcriptional regulation of Mcl-1 has been highly studied to demonstrate that Mcl-1 transcription is regulated by several different transcription factors. In studying the overlap between proteins EDD interacts with and those that regulate Mcl-1 transcription, β-catenin, E2F1, and GATA-2 were identified. β-catenin and hypoxia-inducible factor 1 α cooperate in prostate cancer cells as a result of platelet-derived growth factor stimulation to increase *mcl-1* transcription (Iqbal, Zhang et al. 2012). EDD has been identified to ubiquitinate and therefore stabilize β-catenin to promote its activity (Hay-Koren, Caspi et al. 2011). It stands to reason that EDD overexpression in cancer could increase ubiquitination of β-catenin, which results in enhanced *mcl-1* transcription. The knockdown of EDD increased protein expression of the E2F transcription factor 1, which can decrease *mcl-1* transcription (Croxton, Ma et al. 2002, Croxton, Ma et al. 2002, Munoz, Saunders et al. 2007). Thus, overexpression of EDD in cancer could decrease E2F1 expression, allowing *mcl-1* transcription. Furthermore,

transcription factor software identified several transcription factors, such as GATA-2, that regulate *mcl-1* transcription (TFSEARCH). As mentioned above, EDD acts as a transcriptional co-activator for the progesterone receptor, and this receptor also interacts with GATA-2 in breast cancer cells (Henderson, Russell et al. 2002, Magklara and Smith 2009). This suggests that EDD could interact as part of a complex between the progesterone receptor and GATA-2 at the Mcl-1 promoter. Co-immunoprecipitations could be used to identify any of these transcription factors, or other potential transcription factors, as an interacting partner of EDD. Additionally, transfection of one of these transcription factors along with EDD transfection in cells with the *mcl-1* promoter-driven luciferase assay would determine if any of these transcription factors has the potential to cooperate with EDD in order to increase *mcl-1* transcription. In the case of E2F1, transfection of this transcription factor would decrease *mcl-1* transcription, unless overexpression of EDD has an overwhelming function to decrease E2F1 protein expression before it can exert its inhibition of *mcl-1* transcription. Further analysis is needed to determine which transcription factors EDD interacts with, either directly or indirectly as part of a complex, and whether this has an effect on *mcl-1* transcription.

Identify novel targets of EDD ubiquitin ligase activity that are involved in mediating acquired cisplatin resistance

The function of EDD as an E3 ubiquitin ligase is essential for EDD to mediate cisplatin resistance. While a few substrates of EDD's ubiquitin ligase

function have been established, none of these have been established as a direct link between EDD and cisplatin resistance. EDD may promote substrate degradation, as with PAIP2, or it may promote protein stabilization, as with β-catenin as a result of substrate ubiquitination (Yoshida, Yoshida et al. 2006, Hay-Koren, Caspi et al. 2011). In order to determine which substrates are ubiquitinated by EDD, an inducible stable EDD overexpressing cell line would be used to overexpress either wild-type EDD or the ubiquitin ligase defective mutant (Cys2768Ala). In order to create these inducible stable overexpressers, a piggybac transposon-based expression system will be used. Based on a publication from James Rini's lab, the piggyback Rfa plasmid will be used to encode the EDD gene, a transposase plasmid used for cutting and inserting the plasmids into the cell genome, and a PB-RB plasmid to encode the reverse tetracycline-controlled transactivator (rtTA) which includes the tetracycline repressor and the transactivation domain (Li, Michael et al. 2013). This Tet-on system will allow for the addition of doxycycline to cells in order to turn on expression of EDD. Previously, I made these stable cell lines in HEK 293T cells and Cos-1 cells, but very few of the cells express EDD after selection in both puromycin (selects for the Rfa-EDD plasmid) and blasticidin (selects for rtTA expression). To mitigate this problem, I made Rfa plasmids encoding EDD fused to GFP. This will allow for me to select for cells by florescent automated cell sorting (FACS) to isolate only those cells which express either GFP wild-type EDD or GFP Cys2768Ala EDD. Currently, at the time of the writing of this dissertation, HEK293T and HeLa cells transfected with these plasmids have

been used to create these inducible stable EDD overexpressing cell lines as demonstrated in Figure 5.1.

These stable EDD overexpressing cell lines will be used to identify ubiquitinated substrates through an unbiased proteomics screen. Cells will be cultured in either light ($^{12}C_6$-Lysine, $^{12}C_6$-Arginine) or heavy ($^{13}C_6$-Lysine, $^{13}C_6$-Arginine) SILAC media for 6 passages. For example wild type EDD overexpressers would be cultured in light media while the ubiquitin defective mutant EDD overexpressers would be cultured in heavy media. About 6 hours before collection of the cells, the proteasome inhibitor MG132 will be added to allow for the accumulation of ubiquitinated proteins. Trypsin digestion will cut proteins at lysine residues. Immunoprecipitation using an anti-digylcyl lysine antibody will bind exposed lysine residues on ubiquitin chains on peptides. The objective is to identify proteins which are ubiquitinated in the wild type EDD overexpressing cells but not in the Cys2768Ala EDD overexpressing cells. Multiple replicates would be required for this experiment, also switching the SILAC media between wild type and mutant EDD to control for differences in SILAC incorporation, in order to monitor reproducibility of the identified substrates. The basic experimental outline for this is depicted in Figure 5.2.

Once targets of EDD's ubiquitin ligase activity are established by mass spectrometry analysis, these targets would be validated through *in vitro* ubiquitination assays. The targets which may play a role in DNA damage response, cellular survival, and cisplatin sensitivity would be analyzed further through knockdown and overexpression studies. This would allow for conclusions

to be drawn about whether a substrate is involved in mediating cisplatin resistance in ovarian cancer cell lines. It is expected that substrates identified will play a role in DNA damage repair pathways (such as TOPBP1), apoptotic pathways, proliferation pathways (such as downstream effectors of ERK or Akt), transcription (such as β-catenin and the progesterone receptor), translation (such as PAIP2), and cell cycle control (such as katanin). The results from this would elucidate the mechanism by which EDD increases cisplatin resistance in cancer, as examined in aim 2 (Chapter 3).

Figure 5.1.

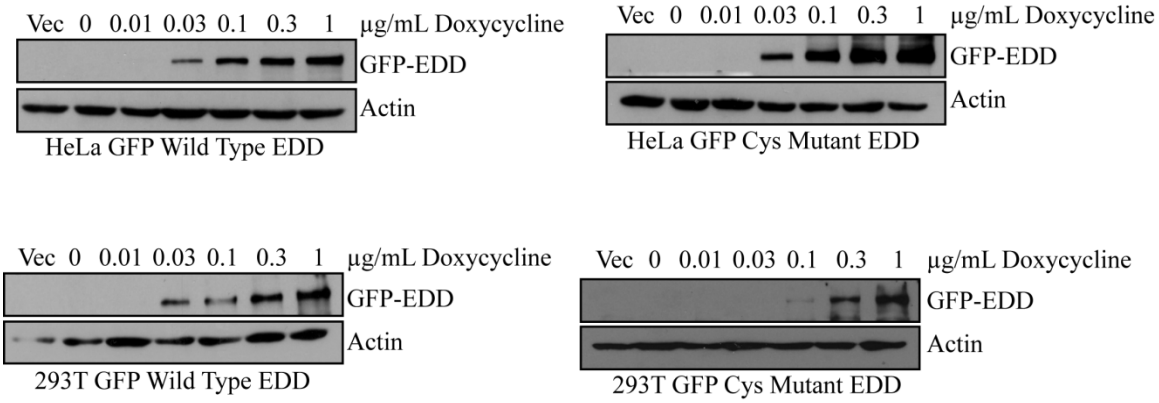


Figure 5.1. Stable GFP-EDD overexpressing cell lines. HeLa and HEK 293T cell lines were transfected with either empty Rfa vector control, GFP-Wild type EDD Rfa, or GFP-Cysteine mutant EDD Rfa vector. These cell line populations were selected with blasticidin and puromycin. Expression of GFP-EDD was induced with different concentrations of doxycycline.

Figure 5.2. (Adapted from http://www.london-research-institute.org.uk/sites/default/files/protein_analysis_and_proteomics/sailac.bmp)

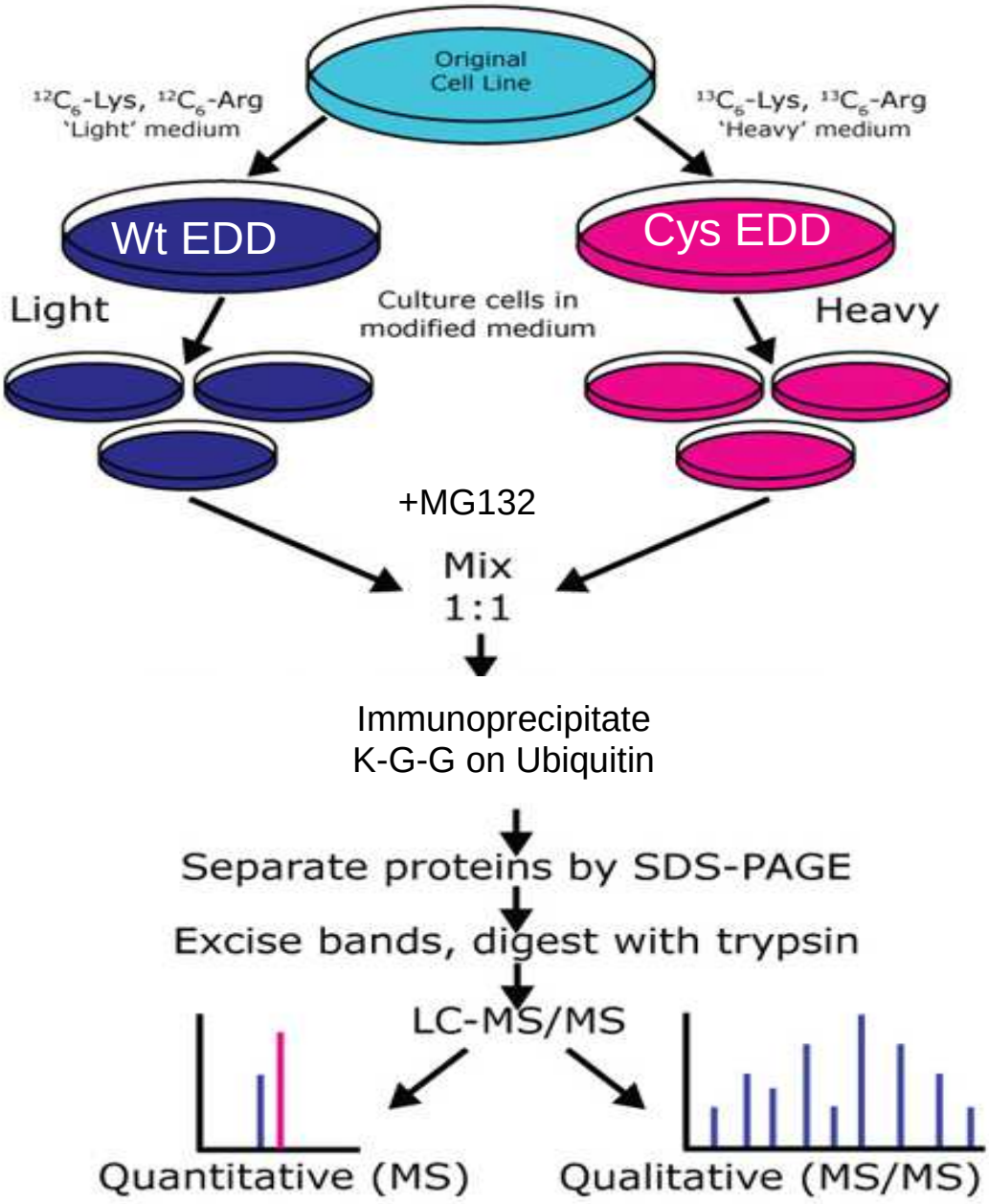


Figure 5.2 Mass spectrometry analysis of ubiquitinated proteins from EDD overexpressing cell lines. Inducible stable overexpression cell lines, both wild type EDD and cysteine mutant of EDD, will be grown in SILAC media, either heavy or light, for 6 passages. MG132 will be added 6 hours before collection. Cell lysates will be mixed together and ubiquitinated proteins will be immunoprecipitated using an antibody to the lysine-glycine-glycine remnant on ubiquitinated proteins after trypsin digestion. Proteins will be separated by SDS-PAGE, excised, and sent for mass spectrometry analysis.

Drug screen for compounds that will enhance cisplatin sensitivity when EDD is overexpressed

Typically, E3 ubiquitin ligases are not easily targeted by small molecule inhibitors due to the lack of a defined catalytic pocket. Rather E3 ubiquitin ligases, such as EDD, rely on protein-protein interactions across multiple domains on the protein allowing for ubiquitin, an E2 enzyme, and a substrate to bind to the ligase, often simultaneously. My dissertation research identifies EDD as an important regulator of cisplatin resistance and cellular survival in ovarian cancer, and potentially oral squamous cell carcinoma. Figure 3.6 demonstrates the value of knocking down EDD with DOPC liposome nanoparticles encapsulating EDD siRNA to increase cisplatin sensitivity in ovarian tumors *in vivo*. SiRNAs are much more difficult to use as a therapeutic than small molecule inhibitors. In order to create a therapy based on this research, a drug screen could be utilized to identify compounds that could either inhibit EDD, which is overexpressed in many cancers, or interact with an interacting partner of EDD in order to prevent EDD's ability to increase cisplatin resistance. In theory, it is likely that one of these drugs that enhances cisplatin sensitivity when EDD is overexpressed would prevent EDD's ubiquitination of an unknown substrate since EDD increases cisplatin sensitivity through its ubiquitin ligase activity.

Utilizing inducible GFP-EDD stable overexpressing cell lines, as detailed above, a combination of cisplatin at a sublethal dose (EC_{10}) and 10 µM of a compound from the ChemBridge library would be used to test for compounds that enhance cisplatin induced apoptosis, as illustrated in Figure 5.3. A

sulforhodamine B cytotoxicity assay (SRB assay) would be used as a high-throughput screen for compounds which increase cisplatin sensitivity in these EDD overexpressing cells as this assay analyzes cell density based on cellular protein content as an indirect measure of cellular survival. Approximately 15,000 compounds from the ChemBridge Library would be screened and the top 0.1% of these drugs along with their chemotypes would be validated. These top compounds would be tested in a variety of assays (MTT assay, propidium iodide staining for apoptosis, and colony formation assays) in EDD overexpressing cells, ovarian cancer cell lines, and primary ascites from MUSC patients, along with cisplatin treatment to determine which compounds are effective at increasing cisplatin resistance. A future aim of this project is to analyze the mechanism which these compounds affect in order to gain a better understanding of the signaling pathways EDD is affecting to mediate cisplatin resistance. It is possible that compounds would be identified that do not modify EDD or its cellular functions, but this approach would still identify compounds which could be useful in patients that are treated with cisplatin as a first line therapeutic or patients which are resistant to cisplatin treatment.

Figure 5.3

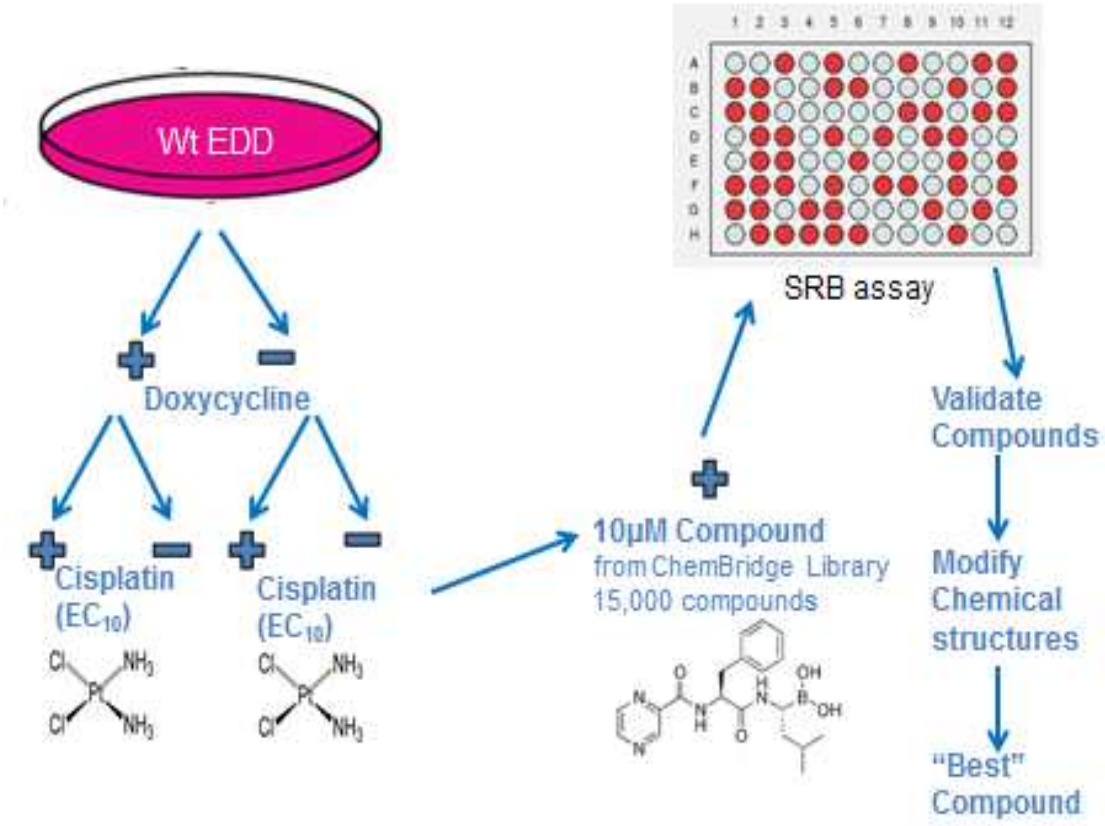


Figure 5.3. Drug Screen for compounds that increase cisplatin sensitivity. Wild type EDD stable overexpressing cells (doxycycline inducible) will be treated with a suboptimal dose of cisplatin along different compounds from the ChemBridge library. Controls used will be those cells without EDD overexpression and cells not treated with cisplatin. A SRB assay will be used to measure cytotoxicity. Compounds will be validated and modified to find the most suitable compound for increasing cisplatin sensitivity.

Analyze alternative responses to stable EDD knockdown in oral squamous cell carcinoma cell lines

Based on results from aim 3 (Chapter 4), it is apparent that oral squamous cell carcinoma cell lines vary in their responses when EDD is knocked down with shRNA. In UM22B cells, as expected based on results seen in ovarian cancer cell lines, the stable knockdown of EDD sensitizes cells to cisplatin. It should be noted that this response only occurred at significant levels at moderate doses of cisplatin treatment (20 μM and 30 μM) as shown in Figure 4.6. Since EDD mediates cisplatin resistance based on its E3 ubiquitin ligase activity, there may be something that EDD ubiquitinates which is responsible for maintaining a fine balance in detecting and/or repairing DNA damage. This unknown ubiquitinated substrate may be affected when there is a moderate level of DNA damage, but DNA damage repair is not effective. As proposed above, it would be valuable to create a better assessment of the pathways and substrates that EDD affects through the identification of its ubiquitin ligase targets.

On the other hand, UM74B cells do not appear to be sensitive to EDD knockdown-induced cisplatin sensitivity, although these cells do exhibit slower rates of proliferation as a result. Based on these results, an analysis of the DNA damage response pathways and cell cycle control pathways as a result of EDD stable knockdown with or without cisplatin treatment would be a useful method to determine the role(s) EDD is playing in this certain oral squamous cell carcinoma cell line. EDD stable knockdown cell lines could be treated with or without cisplatin and lysates from these cells could be evaluated by immunoblot for

altered expression of proteins involved in mediating DNA damage response and cell cycle control. In addition, these stable knockdown cells could be treated with other DNA damaging agents such as carboplatin, gemcitabine, etoposide, and doxorubicin to determine if these chemotherapeutics also decrease the rate of proliferation in these cells when EDD is lost.

It is crucial based on these unexpected results in different oral squamous cell carcinoma cell lines that the number of cell lines used in aim 3 be expanded. This would allow for statistically significant conclusions to be drawn about EDD's role in regulating cellular survival and cisplatin resistance in oral squamous cell carcinoma. Once a role for EDD can be established as regulator of cisplatin resistance in oral squamous cell carcinoma, stable EDD knockdown cell lines can be made and tested in nude mouse xenograft models to confirm these results *in vivo* (Figure 5.4). It is expected that *in vivo* results would be similar to those seen in our ovarian cancer xenograft model in Figure 3.6, which established that knockdown of EDD increased cisplatin sensitivity.

Figure 5.4

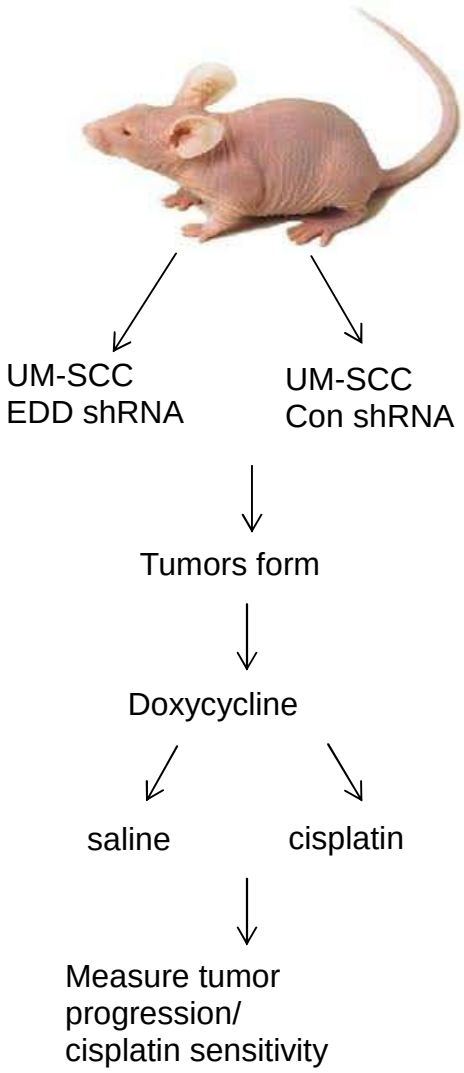


Figure 5.4. *In vivo* model to test increased cisplatin sensitivity due to EDD stable knockdown. Nude mice will be injected orthotopically with UM-SCC oral squamous cell carcinoma cell lines expressing either EDD shRNA or scrambled control shRNA. Once tumors are established, doxycycline will be added to the normal mouse chow to induce expression of the shRNA in each tumor. Mice will then be treated with either saline control or cisplatin and tumor progression will be measured to determine cisplatin sensitivity.

Establish the role of EDD phosphorylation

While the focus of this dissertation is the mechanisms by which EDD affects cellular survival and cisplatin resistance, it is critical to gain a better understanding of what regulates EDD. One such method of regulation of EDD is phosphorylation by ERK2 (Eblen, Kumar et al. 2003). Our lab has also published mass spectrometry results identifying 24 sites of phosphorylation (Bethard, Zheng et al. 2011). It is currently unknown which kinases, other than ERK2, contribute to these sites of phosphorylation. Moreover, it is unknown how phosphorylation of EDD regulates its protein stability, localization, and cellular functions such as ubiquitin ligase activity and protein-protein interactions. To gain a better understanding of these effects, the known sites of phosphorylation of EDD could be mutated to alanine to mimic a lack of phosphorylation at any single site or a combination of phosphorylation sites. Based on the sites of phosphorylation, consensus sites of kinases could be analyzed to determine which kinases may be involved in mediating phosphorylation. An *in vitro* kinase assay could then be used to test these kinases to determine if they can phosphorylate EDD on these sites, but not when these sites are mutated to alanine. Once potential kinases are established that phosphorylated EDD, kinase inhibitors could be used to validate phosphorylation by these kinases. These inhibitors could also establish how phosphorylation of EDD is required to establish protein-protein interactions. These interactions allow for EDD's activity as an ubiquitin ligase and as a transcriptional co-factor, such as for the transcriptional regulation of *mcl-1*. It is also possible that a kinase inhibitor could

be used in conjunction with cisplatin treatment if phosphorylation of EDD by this kinase is required for ubiquitin ligase activity, thus the kinase inhibitor would sensitize cells to cisplatin.

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