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GEBZE TECHNICAL UNIVERSITY
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A THESIS OF DOCTORATE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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GEBZE TEKNİK ÜNİVERSİTESİ
LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

ERCC1'İN DNA HASAR YANITI VE HÜCRE SAĞKALIM KONTROLÜNDEKİ İŞLEVİNİN ARAŞTIRILMASI

ŞULE ERDEMİR SAYAN

DOKTORA TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK
ANABİLİM DALI

DANIŞMAN: PROF. DR. TAMER YAĞCI

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MEMBER (THESIS ADVISOR)	: Prof. Dr. Tamer YAĞCI
MEMBER	: Prof. Dr. Uygar Halis TAZEBAY
MEMBER	: Dr. Öğr. Üyesi Zeynep TOKCAER KESKİN
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To my family,

ABSTRACT

DNA damage recognition-repair or –apoptosis is a critical feature of metazoan biology that is necessary to eliminate the possibility of creating genetically defective progeny. These pathways are also essential in removing somatic cells that received extensive DNA damage by external factors such as UV or internal insults such as reactive oxygen species. Importantly, sensing the extent of DNA damage is critical for a cell to coordinate an action towards DNA repair or apoptosis. For those reasons, it is not unexpected to recognize DNA damage recognition-repair pathways feeding information to apoptosis signaling in higher eukaryotes. Certain DNA damage recognition-repair pathways such as homologous recombination (HR) or non-homologous end joining (NHEJ) are not present in prokaryotes. They were evolved along with eukaryotic organisms, therefore have the ability to signal a cell to undergo apoptosis when DNA damage deemed unrepairable. Among all other DNA repair pathway proteins, only ERCC1-XPF complex, a member of nucleotide excision repair (NER) pathway also take part in higher eukaryote specific cellular events such as class switch recombination and telomere maintenance but has not been implicated in apoptosis signaling before. Therefore, we hypothesized a possible role of NER pathway proteins at the initiation or pre-mitochondrial phase of apoptosis. We have taken an empirical approach and analysed the expression of all components of NER complex proteins upon DNA damage induced or death receptor mediated apoptosis by western blotting. We identified the abundance of ERCC1, XPA and XPC proteins changing with the extent of pro-apoptotic stimuli, ERCC1 being the unique component that is decreasing during DNA damage induced but not upon death receptor ligation by TRAIL. ERCC1 protein has been depicted as a critical component of NER machinery with its absence making the biggest impact on sensitisation of cells to UV induced apoptosis. Our concentration and kinetic experiments registered ERCC1 decrease as an early event in doxorubicin or oxaliplatin induced apoptosis, happening before mitochondrial depolarisation, as assessed by western blotting and flow cytometry. Further, stabilization of ERCC1 using proteasome inhibitors such as MG132 or Bortezomib rendered cells resistant to DNA damage induced apoptosis. These results explain why clinically relevant proteasome inhibitors such as Bortezomib yielded negative results as part of a combination chemotherapy. Understanding the role of ERCC1 in apoptosis is critical for cancer therapy as DNA damage induced by commonly used chemotherapeutic agents such as cisplatin, oxaliplatin or mitomycin C is primarily repaired by NER machinery. Additionally, the use of proteasome inhibitors or other ancillary therapeutic agents along with conventional chemotherapy requires deep knowledge of DNA damage recognition-repair-apoptosis linking pathways.

Keywords: Nucleotide Excision Repair, Apoptosis, ERCC1, XPA, XPC, Proteasome-Dependent Degradation.

ÖZET

DNA hasarı tanıma-onarımı veya –apoptoz, metazoan biyolojisinin genetik olarak kusurlu nesiller yaratma olasılığını ortadan kaldırmak için gerekli olan kritik bir özelliğidir. Bu yolaklar aynı zamanda UV gibi dış etkenler veya reaktif oksijen türleri gibi hücre içi etkiler nedeniyle yoğun DNA hasarına uğrayan somatik hücrelerin uzaklaştırılmasında da gereklidir. Daha da önemlisi, DNA hasarının boyutunu algılamak, bir hücrenin DNA onarımı veya apoptoz yolaklarını koordine edebilmesi için kritik öneme sahiptir. Bu nedenlerden dolayı, yüksek ökaryotların apoptoz sinyal yolaklarına bilgi sağlayan DNA hasarı tanıma-onarım yolaklarına sahip olması beklenmedik bir durum değildir. Homolog rekombinasyon (HR) veya homolog olmayan uç birleştirme (NHEJ) gibi belirli DNA hasarı tanıma-onarım yolları prokaryotlarda mevcut değildir. Ökaryotik organizmalarla birlikte oluşmuş ve evrimleşmişlerdir; bu nedenle, DNA hasarının onarılamaz olduğu düşünüldüğünde bir hücreye apoptoza gitmesi için sinyal verme yeteneğine sahiptirler. Diğer tüm DNA onarım yolaklarında yer alan proteinler arasında yalnızca nükleotid eksizyon onarımı (NER) yolağının bir üyesi olan ERCC1-XPF kompleksi, sınıf değiştirme rekombinasyonu (CSR) ve telomer bakımı gibi daha yüksek ökaryot spesifik hücresel olaylarda da yer alır ancak daha önce apoptoz sinyal yolağında rol aldığı gösterilmemiştir. Bu nedenle, NER yolağı proteinlerinin apoptozun başlangıcında veya mitokondri depolarizasyonu öncesi aşamada olası bir rolü olabileceğini varsaydık. Ampirik bir yaklaşım benimsedik ve NER kompleks proteinlerinin tüm bileşenlerinin DNA hasarının neden olduğu veya ölüm reseptörünün aracılık ettiği apoptoz üzerine ekspresyonunu western blot yoluyla analiz ettik. ERCC1, XPA ve XPC proteinlerinin miktarının, pro-apoptotik uyaranların kapsamına göre değiştiğini belirledik ve ERCC1 proteinin, TRAIL ligantının ölüm reseptörlerine bağlanması sonrasında oluşan apoptoz sonucu değil sadece indüklenen DNA hasarı sonrasında oluşan apoptoz sırasında azaldığını gözlemledik. ERCC1 proteini, NER mekanizmasının kritik bir bileşeni olarak tasvir edilmiştir ve yokluğu, hücrelerin UV kaynaklı apoptoza duyarlı hale gelmesi üzerinde en büyük etkiyi yapmaktadır. Western blot ve akış sitometrisi ile değerlendirdiğimiz konsantrasyon ve kinetik deneylerimizin sonucu, doksorubisin veya oksaliplatin kaynaklı apoptozda ERCC1 düşüşünün mitokondriyal depolarizasyondan önce meydana gelen erken bir olay olduğunu gösterdi. Ayrıca, MG132 veya Bortezomib gibi proteazom inhibitörleri kullanarak ERCC1 proteinini stabilize hale getirdik ve bunun hücreleri DNA hasarına bağlı apoptoza karşı dirençli hale getirdiğini gözlemledik. Bu sonuçlar, Bortezomib gibi klinik açıdan anlamlı proteazom inhibitörlerinin, kombinasyon kemoterapisinin bir parçası olarak neden olumsuz sonuçlar verdiğini açıklamaktadır. Sisplatin, oksaliplatin veya mitomisin C gibi yaygın olarak kullanılan kemoterapötik ajanların neden olduğu DNA hasarı öncelikle NER proteinleri tarafından onarıldığından, ERCC1'in apoptozdaki rolünü anlamak kanser tedavisi için kritik öneme sahiptir. Bununla birlikte, proteazom inhibitörlerinin veya diğer yardımcı terapötik ajanların geleneksel kemoterapiyle birlikte kullanılabilmesi için DNA hasarını tanıma-onarım-apoptoz bağlantı yolakları hakkında daha fazla bilgi edinmek gereklidir.

Anahtar Kelimeler: Nükleotid Eksizyon Tamiri, Apoptoz, ERCC1, XPA, XPC, Proteozoma Bağlı Degredasyon.

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LIST OF SYMBOLS AND ABBREVIATIONS

ΔΨm	:	Mitochondrial membrane potential
µg	:	Microgram
µl	:	Micro liter
6-4 PP	:	6-4 pyrimidine-pyrimidone photo-product
8-oxo-dG	:	8-Oxo-7,8-dihydro-2'-deoxyguanosine
9-1-1	:	Rad9-Rad1-Hus1
AP	:	Apurinic/apyrimidinic
AIF	:	Apoptosis-inducing factor
APAF-1	:	Apoptotic protease activating factor 1
APS	:	Ammonium persulfate
ATCC	:	American Type Culture Collection
ATM	:	Ataxia-telengiectasia mutate
ATR	:	ATM and Rad3 related
ATRIP	:	ATR interacting protein
bp	:	Base pair
BPB	:	Bromophenol blue
BER	:	Base excision repair
BRCA1	:	Breast cancer protein 1
BTZ	:	Bortezomib
cDNA	:	Complementary DNA
CHK1	:	Checkpoint kinase 1
CHK2	:	Checkpoint kinase 2
COFS	:	Cerebro-oculo-facio-skeletal syndrome
CPD	:	Cyclobutane pyrimidine dimer
CS	:	Cockayne syndrome
CSR	:	Class switch recombination
DDB1	:	Damage-specific DNA binding protein 1
DDB2	:	Damage-specific DNA binding protein 2
DDR	:	DNA damage response
DISC	:	Death-inducing signaling complex
DMEM	:	Dulbecco's Modified Eagle Medium
DNA	:	Deoxyribonucleic acid
DNA-PK	:	DNA-dependent protein kinase
DMSO	:	Dimethyl sulfoxide
DSB	:	Double-strand break
dsDNA	:	Double-stranded DNA
EDTA	:	Ethylenediaminetetraaceticacid
ERCC1	:	Excision repair cross-complementing group 1
ERCC4	:	Excision repair cross-complementing group 4
FA	:	Fanconi Anemia
FADD	:	Fas-associated death domain
FBS	:	Fetal Bovine Serum
GCO	:	Global Cancer Observatory
GG-NER	:	Global genomic nucleotide excision repair
Gln	:	Glutamine
HhH	:	Helix-hairpin-helix
HR	:	Homologous recombination

HRP	:	Horseradish peroxidase
IAP	:	Inhibitors of apoptosis proteins
ICL	:	Interstrand crosslink
IHC	:	Immunohistochemistry
IMS	:	Inter-membrane space
IR	:	Ionizing radiation
kD	:	Kilodalton
Leu	:	Leucine
MG132	:	Carbobenzoxy-Leu-Leu-leucinal
ml	:	Mililiter
MGMT	:	Methylguanine DNA methyltransferase
MMEJ	:	Microhomology-mediated end-joining
MMR	:	Mismatch repair
MRN	:	Mre11-Rad50-Nbs1
NaCl	:	Sodium chloride
NER	:	Nucleotide excision repair
NHEJ	:	Non-homologous end joining
NSCLC	:	Non-small cell lung cancer
PBS	:	Phosphate Buffered Saline
PARP	:	poly(ADP-ribose) polymerase
PCNA	:	Proliferating cell nuclear antigen
PCR	:	Polimerase chain reaction
Phe	:	Phenylalanine
PI	:	Propidium iodide
PIKK	:	Phosphatidylinositol 3-kinase-related kinase
RFC	:	Replication factor C
RNA	:	Ribonucleic acid
ROS	:	Reactive oxygen species
RPA	:	Replication protein A
RT-PCR	:	Reverse transcriptase polymerase chain reaction
SDS	:	Sodium dodecyl sulfate
SDS-PAGE	:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	:	Small interfering RNA
SSA	:	Single-strand annealing
SSBR	:	Single-strand break repair
ssDNA	:	Single-stranded DNA
TAE	:	Tris/Asetic acid/EDTA
TBS	:	Tris Buffered Saline
TBS-T	:	Tris buffered Saline with Tween 20
TC-NER	:	Transcription-coupled nucleotide excision repair
TEMED	:	Tetramethylethylenediamine
TLS	:	Translesion synthesis
TMRE	:	Tetramethylrhodamine, ethyl ester
TNF-α	:	Tumor necrosis factor alpha
TRAIL	:	TNF related apoptosis inducing ligand
TRF2	:	Telomeric repeat-binding factor 2
TRIS	:	Hydroxymethylaminomethane
TTD	:	Trichothiodystrophy
UV	:	Ultraviolet

XP	:	Xeroderma Pigmentosum
XRCC1	:	X-ray repair cross-complementing protein 1
zVAD-fmk		Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone

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1. INTRODUCTION

1.1. Cancer

Cancer is a major health concern and a prominent cause of premature death worldwide. According to Global Cancer Observatory (GCO), 19.3 million new cancer cases were diagnosed and almost 10 million cancer deaths reported in 2020 and, the estimated number of 28.4 million new cancer cases expected to occur in 2040. Despite the recent advances in treatment, cancer remains the first or second cause of death in most countries. In 2020, breast cancer was the most diagnosed cancer type with 11.7% of new cases, followed by lung cancer (11.4%). However, lung cancer is the leading cancer type in terms of mortality with 18% of all cancers, followed by colorectal carcinoma (9.4%). Cancer significantly burdens healthcare systems in many countries, especially lower-income ones [1]. These indicators show the necessity to discover new and/or more effective treatments.

As outlines in the hallmark articles from Prof. Weinberg and his colleagues, human cells require the acquisition of at least eight unique functional capacities to develop malignant tumors: sustaining proliferative signaling, eluding growth suppressors, resisting cell death, enabling replicative immortality, activating the angiogenesis process, stimulating invasion and metastasis, altering cellular metabolism and escaping from immune attacks [2, 3]. However, individual tumors and tumor types vary in the degree, mechanisms, and co-occurrence of changes in these pathways [4].

The primary characteristic of cancer is accumulation of mutations caused mostly by environmental or hereditary factors. Hence the susceptibility to develop cancer increases dramatically with age.

1.2. Apoptosis

Resistance to cell death or apoptosis is one of the hallmarks of cancer [3]. Apoptosis is a type of programmed cell death that has been evolved by metazoan organisms and occurs in a variety of physiological and pathological circumstances to eliminate cells. The most prominent morphological and molecular signs of apoptosis include membrane blebbing, nuclear DNA fragmentation, and cytoplasmic shrinkage [5]. An

excessive amount of cell death can lead to diseases like Alzheimer's, Parkinson's, or rheumatoid arthritis. In contrast, uncontrolled cell growth and deficit of cell death can cause diseases like cancer [6]. Although the physiological cell death concept was first put forward by Carl Vogt in 1842 due to his observations in the notochord of frog, apoptosis as a term was first used in Kerr *et al.* in 1972 [7, 8]. Studies on the development of nematode, *Caenorhabditis elegans*, enlightened us about apoptosis process in mammalian cells. During the development of the adult worm, 1090 somatic cells are formed, and among them, 131 cells undergo apoptosis at specific points of the process, and this shows that there is astonishing precision and regulation in this mechanism. Since then, apoptosis—a unique and significant form of "programmed" cell death—has been acknowledged and accepted which includes the genetically controlled disposal of unwanted cells [9].

Recognizing that cell death, mostly via apoptosis, plays a major role in both controlling tumor formation and response to therapy has been one of the most significant developments in cancer research in the last two decades. Induction of apoptotic pathways to eliminate cancer cells by chemotherapy, γ-irradiation, or immunotherapy has been adopted by clinical oncology [10].

Apoptosis may initiate as a response to either intrinsic stimuli via the mitochondrial signaling pathway or extrinsic stimuli through cell surface death receptors such as Fas (CD95/APO-1), TNF-α (tumor necrosis factor-α), and TRAIL (TNF related apoptosis inducing ligand) receptors (**Figure 1.1**) [11].

Independent of the initiating stimulus, both pathways converge on the same terminal executioners, caspases. Caspases (cysteine-dependent aspartate-directed proteases), a highly conserved family of proteases, synthesized as inactive zymogens and that are converted into their active forms via proteolytic cleavage. Thirteen mammalian caspases have been recognized and divided into main two groups based on their function: apoptotic and inflammatory caspases. Caspase-1, -4, -5, -11, and -12 are members of the inflammatory caspase family. Apoptotic caspases are further categorized into initiators, including caspase-2, -8, -9, and -10, and effectors such as caspase-3, -6, and -7 [12, 13]. On the other hand, caspase-14 is not categorized as an apoptotic or inflammatory caspase. It is known to participate in the terminal differentiation of keratinocytes [14].

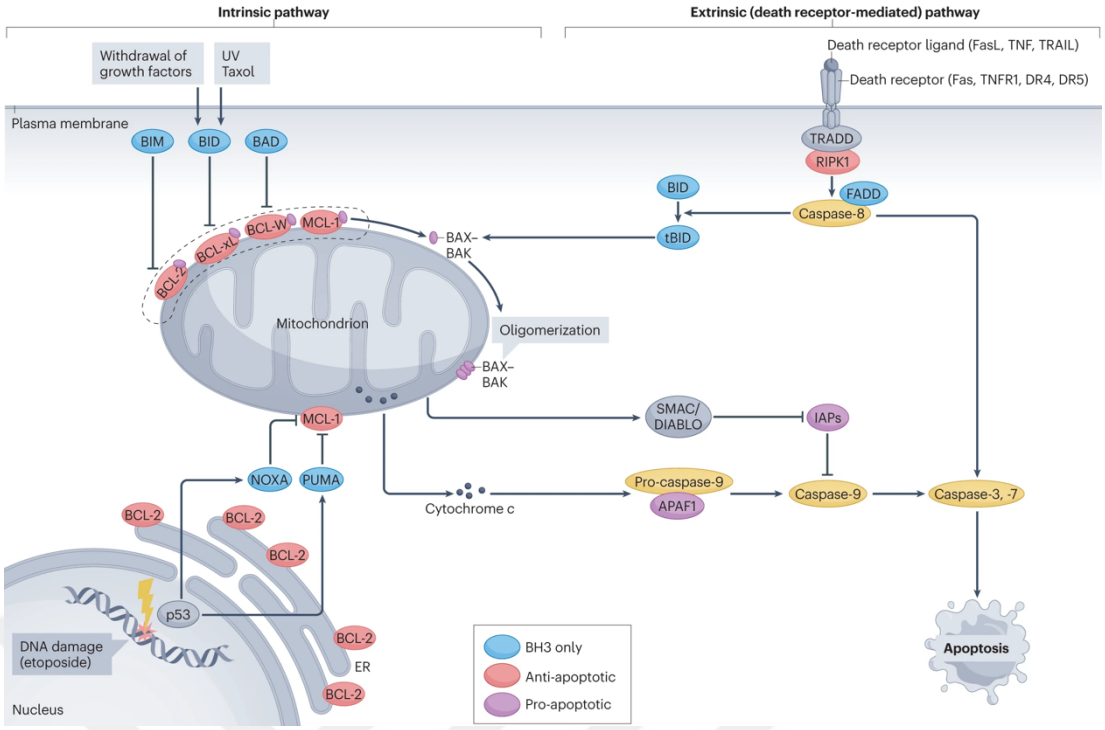

Figure 1.1: Intrinsic and extrinsic apoptosis pathways [15].

Intrinsic (mitochondrial) apoptosis pathway is regulated by a group of proteins from Bcl-2 family. Whilst some members in this family such as Bcl-2, Bcl-w, Bcl-X_L and Mcl-1 show anti-apoptotic properties, others such as Bax, Bak or the BH3-only proteins Bad, Bid, Bim, Puma, Noxa, Bik function as pro-apoptotic [16]. First, stimuli activate oligomerization of Bax and Bak and this event causes disruption of outer membrane of mitochondria and eventually leads to the release of a set of proteins, which are normally found in inter-membrane space (IMS) including cytochrome c, Smac/DIABLO, HtrA2/Omi, AIF (apoptosis-inducing factor) and endonuclease-G. Cytochrome-c binds to Apaf-1 (apoptotic protease activating factor 1) and induces formation of apoptosome complex and Apaf-1-mediated caspase-9 activation. Caspase-9 cleaves and activates effector caspases. Whereas Smac/DIABLO and HtrA2 contribute the process by preventing IAPs (inhibitors of apoptosis proteins) from inhibiting the caspases, endonuclease-G and AIF induce caspase-independent DNA fragmentation [11, 17, 18]

The initiation event in the extrinsic (death receptor) apoptosis pathway requires ligation of the members of the tumor necrosis factor (TNF) receptor gene superfamily. These members have similar, cysteine-rich extracellular domains and a cytoplasmic domain of about 80 amino acids called ‘death domain’, which is important in transfer

the death signal from the cell's surface to intracellular signaling pathways. The best-defined death receptors are Fas, TNF receptor 1, TNF-related apoptosis-inducing ligand-receptor 1 (TRAIL-R1) and TRAIL-R2. When these receptors bind to their ligands (CD95L, TNFα, TRAIL, etc.) receptors' death domains cluster and recruit adaptor proteins such as Fas-associated death domain (FADD). FADD associates with procaspase-8 and forms death-inducing signaling complex (DISC), which results in the activation of caspase-8, and this triggers the activation of downstream effector caspases subsequently [19, 20].

1.3. DNA Damage

The stability and integrity of the genome in a cell is threatened by endogenous or exogenous sources throughout its life. A single cell can be exposed to spontaneous DNA-damaging events up to 10^4-10^5 times per day. Survival of the cell and passing its genetic information to progeny depend on a full-functioning DNA damage recognition and repair machinery [21, 22].

Cancer cells need to evolve quickly to adopt harsh environments such hypoxia therefore they need to retain a higher-than-normal rate of DNA mutations. In that respect, it is not a surprise that critical genes involved in the control of DNA repair/ DNA damage response are mutated in cancers.

Genomic insults are originated from two main sources: exogenous sources such as ultraviolet (UV) radiation, ionizing radiation (IR) (e.g., X-rays), and various genotoxic chemicals (e.g., platinum-based drugs), and endogenous sources including hydrolysis of nucleotide residues, oxidation, alkylation, reactive oxygen species (ROS). As shown in **Table 1.1**, different DNA damages cause different DNA lesions that trigger the DNA damage response and lesion specific-DNA repair pathways in the cell [23].

Table 1.1: DNA insults, origin of damages, and related DNA repair pathways.

DNA insult	Origin of damage	DNA repair pathway
8-oxo-dG / Thymine glycol	ROS/ respiration	Base excision repair (BER)
Abasic site / Uracil, (Hypo)Xanthine	Spontaneous hydrolysis / Spontaneous deamination	Base excision repair (BER)
Mismatches	Replication errors	Mismatch repair (MMR)
Small insertion / deletions	Replication slippage	Mismatch repair (MMR)
Single-strand breaks	IR, ROS	Base excision repair (BER) / Single-strand break repair (SSBR)
Double-strand breaks	IR, ROS, VDJ-recombination	Homologous Recombination (HR) / Non-Homologous End Joining (NHEJ)
Cyclobutane pyrimidine dimers (CPD) 6-4 pyrimidine-pyrimidone photoproducts (6-4 PP)	UV from sunlight	Nucleotide excision repair (NER)
Bulky adducts	Polycyclic aromatic hydrocarbons	Nucleotide excision repair (NER)
Intrastrand / Interstrand crosslinks	Chemotherapy	Nucleotide excision repair (NER) / Homologous Recombination (HR) / Fanconi Anemia (FA) pathway

1.4. DNA Damage Response

To deal with various genotoxic insults, cells developed a system called DNA damage response (DDR) including DNA repair pathways, DNA damage tolerance mechanisms, and cell-cycle checkpoint pathways to maintain genomic integrity and ensure survival of the organism. Depending on the extent of DNA damage, cells may be eliminated by apoptosis, rendered inactive by triggering cellular senescence or they can survive and continue cell cycle progression after checkpoint termination. Impaired DDR system may result in several diseases, such as neurodegenerative disorders, premature ageing, and cancer [24]. DDR components can be grouped under four types of protein: damage sensors, signal transducers (apical PIKKs - phosphatidylinositol 3-

kinase-related kinases - and downstream checkpoint kinases), mediators/adaptors, and effectors (**Figure 1.2**) [25, 26].

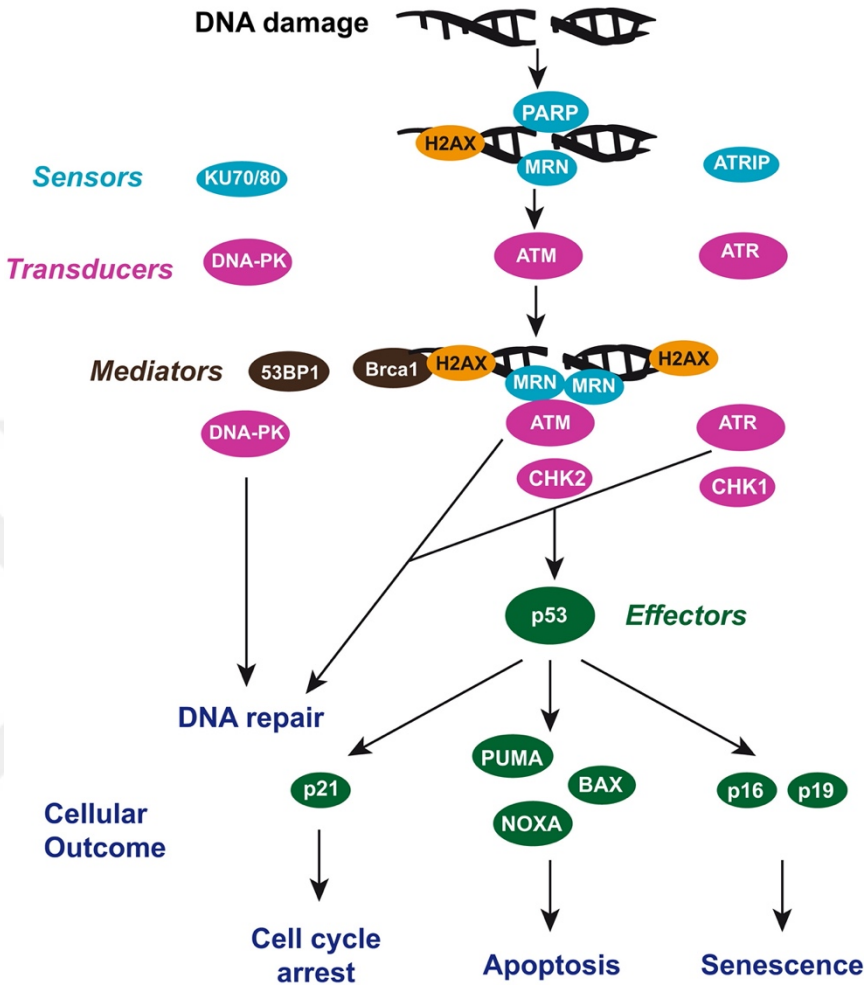

Figure 1.2: DNA damage response pathways [27].

ATM (ataxia-telangiectasia mutated), ATR (ATM and Rad3 related), and DNA-PK (DNA-dependent protein kinase) are members of the family of PIKKs. Whereas ATM and DNA-PK are stimulated in response to double-strand breaks (DSBs), ATR is triggered by single-stranded DNA (ssDNA) or replication stress. Once activated, they initiate a series of phosphorylation events that result in the activation of effector proteins involved in DNA repair, cell cycle or apoptosis [28, 29].

Double-strand breaks in DNA are recognized by Mre11–Rad50–Nbs1 (MRN) complex, which recruits ATM to the damaged site. Activated ATM phosphorylates CHK2 and effector proteins such as p53 through mediator proteins (e.g., BRCA1). Replication protein A (RPA) coated ssDNA triggers the recruitment of ATR and its

partner ATRIP (ATR interacting protein) to the damaged site. RAD9–RAD1–HUS1 (9-1-1) and RAD17–RFC complexes are also required to boost ATR activation. Once activated, ATR phosphorylates its substrates such as CHK1 with the assistance of adaptor proteins (e.g., Claspin). DNA-PK consists of DNA-PKcs (DNA-dependent protein kinase catalytic subunit) and Ku70/Ku80 heterodimer and its activation and recruitment to DSBs is mediated by Ku70/Ku80. Activated DNA-PK demonstrates a major role in non-homologous end joining (NHEJ) pathway for DSB repair [25, 30].

1.5. DNA Repair

Once DNA damage response is triggered, cells can respond by activating specific DNA repair pathways depending on the type of DNA lesion. The DNA repair mechanisms can be divided into seven categories and will be described individually below.

1.5.1. Direct Repair

Direct repair corrects the damaged nucleotides or nucleosides allowing repair without major alterations in the DNA backbone. UV exposure of DNA leads to pyrimidine dimers. An enzyme called DNA photolyase, which is present in all living organisms except placental mammals, binds to DNA and removes the adduct using two chromophore cofactors without disturbing the ribose backbone. Whereas binding of photolyase to DNA is a light-independent reaction, catalysis of the removal of the dimers is initiated by light [31, 32].

On the other hand, another enzyme called methylguanine DNA methyltransferase (MGMT) can be found in all organisms, from bacteria to human cells. MGMT transfers the methyl group at the O^6 site of damaged guanine to its cysteine residues, to repair the damage caused by alkylating agents [26].

1.5.2. Base Excision Repair

Small changes in bases that do not disturb DNA double helix are restored by base excision repair (BER). Several enzymes recognizing specific aberrations e.g., 8-Oxoguanine glycosylase or 3-alkyladenine DNA glycosylase have evolved to function in BER to remove the damaged base. The gap is corrected and filled by DNA

polymerase β and/or DNA polymerase δ/ε, and the nick is sealed by XRCC1/DNA ligase III complex or DNA ligase I depending upon patch size [33].

1.5.3. Mismatch Repair

DNA mismatch repair pathway (MMR) plays a key role in correcting errors made by DNA polymerase or mismatches that occur during replication. MMR pathways comprise following steps: Mismatched nucleotides are detected, and other MMR factors are recruited to the site then mismatch excision is processed and the excised tract is re-synthesised. In human cells, mismatch is recognized by hMutSα (MSH2-MSH6) and hMutSβ (MSH2-MSH3) first, then other components including proliferating cell nuclear antigen (PCNA), replication factor C (RFC), MutLα (MLH1-PMS2 complex), and Exonuclease 1 (Exo1) are recruited to the site. The excised strand is resynthesized by DNA polymerase δ and the gap is sealed by DNA ligase I [34, 35].

1.5.4. Double-Strand Break Repair

DNA strand breaks, especially double-strand DNA breaks (DSB) are the most lethal and difficult to correct. In other DNA repair mechanisms, the unmutated DNA strand acts as a reference point or template. Instead, double strand breaks have no reference and can cause mis-conjugation of unrelated DNA strands, therefore allows chromosomal fissions or chromosomal segregations. This repair mechanism is directly linked with apoptosis pathways. Many chemotherapeutic agents such as doxorubicin, bleomycin, etoposide (VP16) and ionizing irradiation (IR), and certain crosslinking agents at high concentrations such as cisplatin, promote cell death in both normal and cancer cells by creating double strand breaks. DSB repair exists only in eukaryotic cells and is operated by two major mechanisms called as homologous recombination (HR) and nonhomologous end joining (NHEJ) [36]. The type of DSB repair depends on the phase of cell cycle cells are at. A sister chromatid is required for HR and therefore it occurs at S and G2 phases. This type of DSB has relatively high fidelity. NHEJ, on the other hand, predominates at M and G1 stages of cell cycle. NHEJ is a damage limitation exercise for cells but overall can create non-viable progeny. More than 50 proteins take part in this most complicated DNA repair pathway [37]. If these two major repair pathways for DSBs fail to repair, either single-strand annealing (SSA)

or microhomology-mediated end-joining (MMEJ) can be used as a backup mechanism but both these repair systems are error-prone [38].

1.5.5. Interstrand Crosslink Repair

Chemotherapeutic agents or carcinogens such as found in tobacco products can lead to the formation of covalent bonds on the same strand (intrastrand), between two complementary strands (interstrand) or between a base and a protein. These adducts prevent DNA replication or transcription from proceeding. Interstrand crosslinks (ICL) cause the most harm to a cell by inducing double-strand breaks. ICL repair can occur replication-dependent or -independent depending on the cell cycle phase. In both pathways, Fanconi anemia (FA) proteins play the main role and carry out the process by utilising other proteins from different repair pathways such as NER, HR or translesion synthesis (TLS) [39].

1.5.6. Single-Strand Break Repair

During BER pathway, apurinic/apyrimidinic (AP) sites occur. If AP sites are not addressed correctly, they can be recognized as a type of DNA lesion called single-strand breaks (SSB). Then poly(ADP-ribose) polymerases (PARP) 1 and 2 sense SSBs and recruit XRCC1, which functions as a molecular scaffold and interacts with DNA end processing and gap filling proteins to complete the repair [40].

1.5.7. Nucleotide Excision Repair

Mutagenic and carcinogenic agents lead to different base damages in DNA, therefore create torsional stress, are corrected by nucleotide excision. Unlike more simple (prokaryotic) forms of excision repair, which is carried out by UvrA, UvrB and UvrC, *in vitro* studies indicated that 15 polypeptides grouped into six repair factors are required to convey eukaryotic NER pathway effectively. In humans, mutations in NER genes result in Xeroderma Pigmentosum (XP) syndrome, which presents with increased sensitivity to UV and risk of skin cancer. Other associated diseases are Cockayne syndrome (CS) and trichothiodystrophy (TTD) [25, 41]. Details of XP syndromes, Cockayne syndrome and their association with specific NER genes will be discussed in section 1.6.2.

The NER process involves following steps: Damage recognition, dual incision, repair, synthesis, and ligation, respectively. There are 2 types of NER mechanism: global genomic nucleotide excision repair (GG-NER) and transcription-coupled nucleotide excision repair (TC-NER) (**Figure 1.3**). Whilst GG-NER occurs on the lesions over the entire genome, TC-NER take places in the repair of transcription-blocking lesions on a transcribed DNA strand. The damage is sensed by XPC-HHR23B complex in GG-NER process, whereas stalled RNA Polymerase II triggers the recognition in TC-NER and Cockayne syndrome proteins including CSB and CSA translocate RNA Polymerase II to make the lesion available for repair [42]. Certain form of DNA adducts such as cyclobutane pyrimidine dimers (CPDs) do not lead to major helix distortions. In such cases, the XPE complex (DDB1 and DDB2) serves in the initial detection of the lesions and recruits XPC to start GG-NER [43].

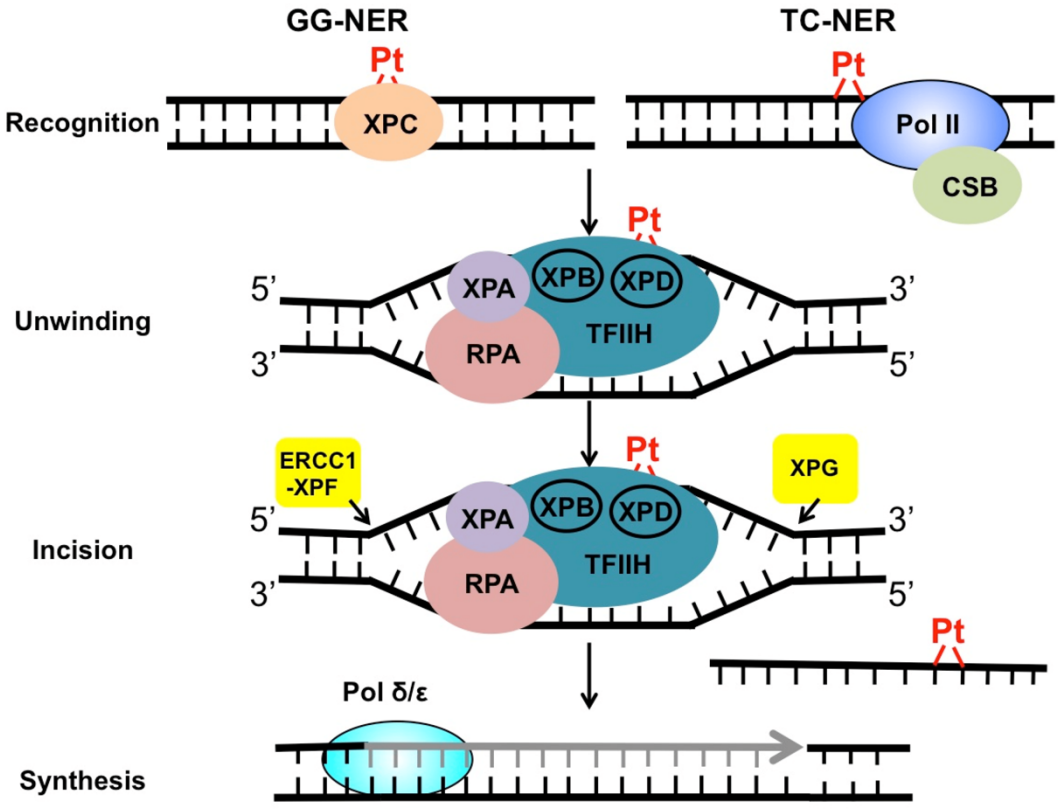

Figure 1.3: Nucleotide excision repair pathways [44].

After damage recognition, both types of NER mechanisms follow the same steps: TFIIH-XPB-XPD helicase complex binds the damaged region and unwinds the distorted DNA. This structure is stabilized by the XPA-RPA complex. Then XPC is

replaced by XPG in the complex and lastly, ERCC1-XPF is recruited. Once the pre-incision complex is formed, the ERCC1-XPF complex makes the 5' incision first, followed by initiation of repair synthesis and then a 3' incision is made by XPG [45]. Excision of 24-32 nucleotides containing the damaged DNA is followed by gap filling process by Pol ε and δ with the help of RFC and PCNA. The gap is subsequently sealed by DNA ligase 1 and XRCC1/DNA ligase III complex in TC-NER and GG-NER, respectively [22, 25, 46, 47].

1.6. ERCC1-XPF Complex

ERCC1 gene, which is located at chromosome 19, is composed of 10 exons, covering more than 70 kilobases (kb) of the genome and encodes the ERCC1 protein of 297 amino acids. *ERCC1* is the first cloned human DNA repair gene [48, 49]. ERCC1 protein associates with XPF to form a heterodimer complex. XPF is encoded by the *ERCC4* gene, which is located at human chromosome 16, and comprises 916 amino acids [50]. Both ERCC1 and XPF are the members of XPF/MUS81 family. Their orthologs in *S. cerevisiae* and *S. pombe* are Rad1-Rad10 and Rad16-Swi10, respectively [51]. Whilst XPF/MUS81 family members exist as homodimers in archaea, they form heterodimeric complexes in higher eukaryotes and only one subunit which exhibits endonuclease activity is enough to function [52].

ERCC1-XPF complex is a structure-specific endonuclease which incises stem-loops, overhangs, bubbles, 3'-flaps and splayed-arm structures [53-55]. XPF contain a helicase-like domain, a central ERCC4 nuclease domain and two tandem helix-hairpin-helix $(HhH)_2$ DNA-binding domains. ERCC1 has also a central ERCC4-like domain without a catalytic motif, which mediates interactions with single-stranded DNA (ssDNA) and XPA in the NER pathway and it is followed by a $(HhH)_2$ domain same as in XPF (**Figure 1.4**). ERCC1 and its partner XPF interact with each other through their $(HhH)_2$ domains for dimerization [56, 57]. In addition to dimerization, helix-hairpin-helix domains of ERCC1 and XPF contribute to binding to double-stranded DNA (dsDNA) and ssDNA, respectively (**Figure 1.5**) [58].

ERCC1 gene undergoes alternative splicing, which leads to 4 different isoforms called 201, 202, 203 and 204. Among them, isoform 202 was reported as the only transcript which functions in NER. The function of other isoforms is still mostly unknown [59, 60].

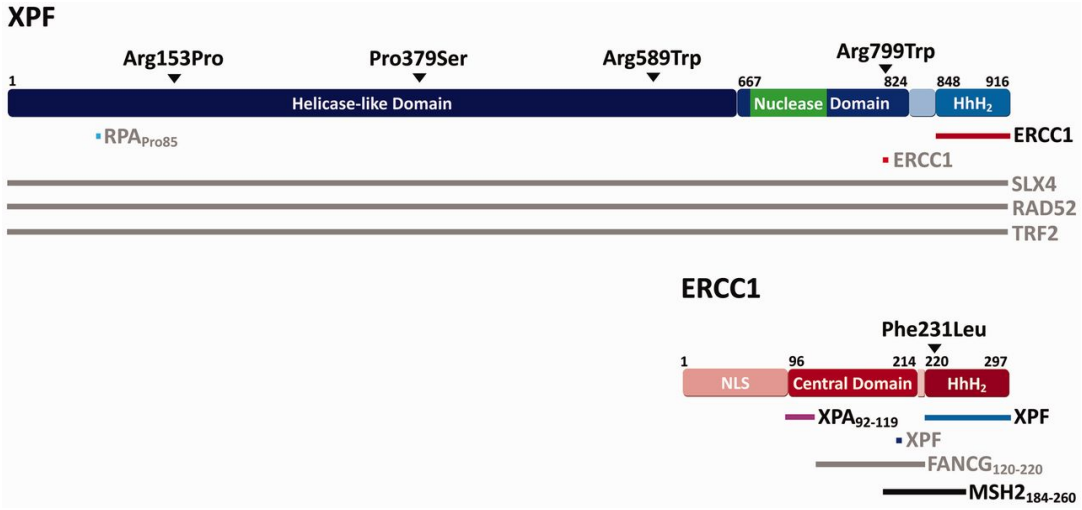

Figure 1.4: Domain structures of ERCC1 and XPF (ERCC4) proteins [61].

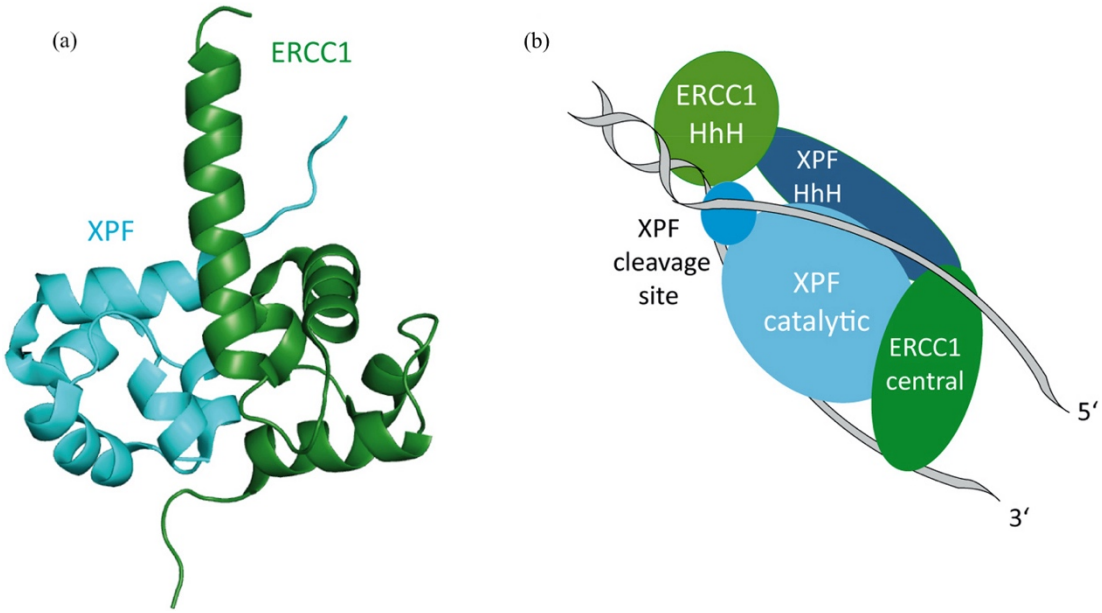

Figure 1.5: Molecular structure and model of ERCC1 with its binding partners. (a) Structure of the human ERCC1-XPF heterodimer. (b) Model of the ERCC1-XPF complex binding to a splayed DNA substrate [62].

1.6.1. Biological Functions of ERCC1-XPF

ERCC1-XPF complex participates not only in the nucleotide excision repair discussed before but also in the repair of DSBs, interstrand crosslinks (ICLs) and in maintaining telomeres [58]. ERCC1-XPF deficient cells are more sensitive to IR exposure, which is a DSB source and the repair level of DSBs decreases. It has been acknowledged that ERCC1-XPF complex function in error-prone SSA mechanism and removes 3-

overhangs in broken ends. On the other hand, the function of ERCC1-XPF in NHEJ is still in debate [63-65]. During V(D)J recombination and class switch recombination (CSR), which occur whilst the development of B and T cells, DSBs form and this kind of DSB repair is carried out by NHEJ. A recent study showed that ERCC1-XPF complex functions as 3' flap endonuclease during an alternative end joining-mediated class switching [66, 67]. ERCC1-XPF deficient cells are also very sensitive to crosslinking agents such as cisplatin and mitomycin C, which can halt DNA replication and transcription [39]. ERCC1-XPF makes the incision and unhooks the crosslink. During this repair process, ERCC1-XPF complex associates with different proteins such as SLX4 and other FA repair pathway proteins [68, 69].

Beyond DNA repair, it is thought that the ERCC1-XPF complex may play a role in the maintenance of telomeres due to co-localizing with a protein called TRF2 (telomeric repeat-binding factor 2). TRF2 stabilizes the DNA loop conformations in the ends of chromosomes. It was reported that inhibition of this protein leads to the shortening of telomeres via removal of the uncapped 3'-overhangs by ERCC1-XPF [70]. Another study showed that ERCC1-XPF controls telomere shortening [71]. Although no direct interaction was shown between them, both TRF2 and ERCC1-XPF has an interaction with SLX4, suggesting that they may interact with each other via SLX4 [72].

1.6.2. Disorders Associated with ERCC1-XPF

The mutations in NER pathway proteins are associated with several disorders. Xeroderma pigmentosum (XP) is one of the well-known NER deficiency diseases, which is characterized by symptoms such as photosensitivity, neurological abnormalities, and susceptibility to skin cancer. The mutations can occur in any NER enzymes of XP family genes (A to G and V) with different degree of severity but there is no ERCC1 mutation associated to XP disorders [38].

Among all XP disorders, XP-F disease results from the mutations in *ERCC4* gene. It occurs less frequently compared to other XP diseases. XP-F patients have decreased XPF nuclease activity due to missense mutations in at least one of ERCC4 allele.

The mutations in the *ERCC4* gene may also result in the development of XFE progeroid syndrome, which causes problems such as premature aging and severe photosensitivity [73].

The other disease related to NER deficiency is Cockayne syndrome, which result from mutations in *ERCC8* (CSA) and *ERCC6* (CSB) genes leads to premature aging, and developmental and neurological abnormalities. Besides, *ERCC1* and *ERCC4* mutations were also reported in CS [74].

ERCC4 is also involved in Fanconi anemia, which is another rare disorder caused by impaired ICL repair. A total of 22 genes have been reported associated with FA, which is characterized by symptoms including bone marrow failure, cancer susceptibility, and developmental and congenital abnormalities [75, 76]. XPF mutations in these patients lead to decreased ICL repair while retaining its function in NER [77].

On the other hand, there is only one disorder was reported concerning ERCC1 mutations called cerebro-oculo-facio-skeletal syndrome (COFS). Additionally, *XPD*, *XPG* and *ERCC6* mutations were reported in COFS. The patients with COFS exhibit severe developmental, skeletal, and facial abnormalities [74].

ERCC1 mutations are extremely rare in humans and the results obtained from ERCC1 mouse models suggest that embryonic lethality can be the cause of this rarity [78]. In line with this, there are only a handful of patients have been reported until now. The first reported patient had a stop codon at Gln158 in the central domain of ERCC1 protein and another mutation at residue 231 (Phe231Leu) in the XPF binding site of ERCC1. The other patient harboured one mutation only which is Phe231Leu. Both patients died in their first 2 years [74, 79].

1.6.3. ERCC1 and ERCC4 Mouse Models

To understand the importance of the ERCC1-XPF complex, several mouse models were developed. To mimic one of the XP-F patients, a mutation was introduced in exon 8 of XPF, generating a truncated protein. These homozygous XPF mutant mice, which were born with Mendelian frequency, had no detectable XPF in their tissues and they died by 3 weeks of age and were extremely underweight compared to wild-type mice [80].

Several ERCC1 knock-out or mutant mouse models have been generated. In general, ERCC1-deficient mice exhibit several symptoms such as premature aging, abnormalities and malfunction in the liver and kidney, fast turnover of hematopoietic cells, and neurodegeneration. Their cells are also more sensitive to IR or crosslinking agents [58, 81]. There are 2 knock-out models, which were created by inserting a neomycin resistance cassette into exons-5 or -7, resulting in a truncated ERCC1 protein. The exon 5 disrupted mice were born in line with Mendelian frequency and the exon 7 disrupted mice were born with sub-Mendelian frequency. However, all homozygous ERCC1 deficient pups were severely runted and died within 3 weeks of birth. This ERCC1 null mouse phenotype is very similar to mutant ERCC4 mouse models, suggesting that they function in a complex [78, 82]. Another generated mouse model of ERCC1 had 7 amino acid deletions from the C-terminus of the protein in the XPF binding region at position 292 ($ERCC1^{*292}$ or $ERCC1^{\Delta/\Delta}$). The mutant protein expression was able to be detected in the tissues of mutant mice. These mutant litters lived until 6 months of age due to detectable repair activity compared to ERCC1 null mice [78]. Moreover, another mouse model, which carries the combination of a null and a mutant ERCC1 ($ERCC1^{-/\Delta}$) was created. These mice lived up to 6-7 months of age and developed several diseases associated with aging in humans such as osteoporosis, intervertebral disc degeneration, and neurodegeneration [83-85]. It is commonly used as an ageing model.

1.6.4. ERCC1 Expression and Chemotherapy Resistance

Among all NER proteins, ERCC1 attracts attention most as it is involved in multiple DNA repair pathways such as NER, ICL and DSB [46, 60]. Curiously, ERCC1 does not have a direct enzymatic function but, XPF, its partner is the 5'- endonuclease in the NER system. Since there is no catalytic activity without dimerization, ERCC1 is believed to be essential for the complex to be recruited to the 5' incision site and function [61, 86]. Also, quite interestingly, systemic studies showed that, among all NER pathway components, ERCC1 deficiency causes the highest sensitivity to DNA crosslinking agents, much more than observed when XPF is knocked down by siRNA [87-89]. From such studies, it is evident that ERCC1 has functions other than linking XPF and damaged DNA. Ovarian, bladder and lung cancer cell lines that exhibit resistance to platinum derivatives have been shown to overexpress ERCC1 and possess

enhanced NER activity [90-92]. The hypersensitivity of testicular cancer to platinum-based chemotherapy is also associated with low abundance of ERCC1 [93]. Additionally, patients with advanced metastatic bladder cancer, oesophageal cancer, or advanced gastric cancers, who showed low ERCC1 mRNA and protein expression in their tumors, responded to chemotherapy better and had longer overall survival [94-97]. Moreover, polymorphism studies have been carried out for ERCC1 to associate with response to chemotherapy [98]. A study showed that having the T variant at codon N118N in one of the *ERCC1* alleles was associated with shorter progression-free survival and shorter overall survival in colorectal cancer patients treated with chemotherapy [99]. Another study showed that chemotherapeutic toxicity can be increased by interfering with the NER pathway in the cisplatin resistant OVCAR10 cell line using antisense ERCC1 RNA [100].

In early clinical trials, high ERCC1 expression has also been associated with cisplatin resistance in ovarian and non-small cell lung cancers [101-103]. ERCC1 protein expression has been extensively used as a biomarker for resistance to cisplatin treatment especially in non-small cell lung cancer (NSCLC) [104]. A study reported that patients with low ERCC1 expression in their tumors responded to cisplatin-based therapy better than patients with high ERCC1 expression and low ERCC1 expression was correlated with longer overall survival [105]. The studies in NSCLC are promising but another study suggests that using ERCC1 as a biomarker may lead to different results due to differences between the batches of 8F1 ERCC1 antibody, which is widely used in IHC based clinical trials. It was later understood that this antibody and most of the other tested 15 of ERCC1 antibodies detect not only the full length but also all known four isoforms of ERCC1. Although RT-PCR gives more accurate results than IHC, the ERCC1 protein level seems more important in predicting chemotherapy resistance [59]. Due to IHC-based detection problems, a proximity ligation assay (PLA) based method using monoclonal antibodies against to both functional 202-isoform of ERCC1 and XPF was also developed in a recent study by Kuo *et al.* [106].

ERCC1 is overexpressed in many types of cancer [46] and high ERCC1 protein expression in cancer cells is associated with resistance to apoptosis [107]. However, it has no enzymatic activity like XPF, so it is hard to modulate its function. Hence, targeting the interactions such as between ERCC1 and XPF or ERCC1 and XPA with

small molecules to inhibit ERCC1 may bring forth new possible cancer therapy combinations [61].

2. PURPOSE OF THE STUDY AND ORIGINAL CONTRIBUTION TO KNOWLEDGE

The era of targeted cancer therapeutics has emerged more than 20 years ago; however, cancer patients still need conventional chemotherapy to survive for longer. In that respect, knowing how chemotherapy works, e.g., in which condition cancer cells will die or survive upon genotoxic treatment, holds utmost value. Additionally, strong and valid biomarkers are necessary to guide and predict chemotherapy response. ERCC1 is involved in a variety of DNA repair pathways, therefore the mechanism/s of how ERCC1 controls survival and death is critical to dissect. Importantly, unlike several other key proteins such as ATM, CHK2 or p53, there has been no research in linking ERCC1 function directly to apoptosis and ERCC1 has been proposed as a biomarker only for DNA crosslinking agents such as cisplatin, oxaliplatin and mitomycin C however cancer cells become resistant to a variety of genotoxic insults when ERCC1 is overexpressed [64, 108].

Therefore, it is clear that ERCC1 deficiency is impacting cell death associated with genomic insults more than any other NER component gene. This is reflected to the phenotype of XP gene deficiencies (alive but NER impaired) observed in mice and humans as compared to ERCC1 (embryonic lethal or severely runted). The importance of ERCC1 in DNA damage repair has been studied in detail and ERCC1 overexpressing cells were shown to be resistant to apoptosis. However, these studies never dissected the outcome in a way that allows the discrimination of whether the enhanced DNA repair or a protective activity of ERCC1 against apoptosis as the cause. In another words, there has been no study that eliminated the DNA repair activity of ERCC1, e.g., with a single amino acid substitution and assessed apoptosis resistance to address whether ERCC1 has an intrinsic protective function against apoptosis.

In this study, we aimed to find out the contribution of all NER proteins, including ERCC1, to DNA damage response assessing their protein abundance. To that end, we performed kinetics/concentration assays (using oxaliplatin and doxorubicin), protein stabilization experiments (using MG132 and Bortezomib) to evaluate the resistance to DNA damage-induced apoptosis. Importantly, we assessed the changes in protein abundance of ERCC1 before “the point of no return” in apoptosis in reached to suggest

whether changes in ERCC1 are a cause or effect during apoptosis pathways. Therefore, the mechanism by which ERCC1 protein levels are controlled upon genotoxic stress and how this translates apoptosis-DNA repair switch are important and unique aspects of this thesis. Our findings may impact timing, dosage, and combination of chemotherapy for the benefit of cancer patients. Therefore, this thesis potentially presents significant socio-economical value, after deeper understanding the mechanism that controls ERCC1 stability, pre-clinical and clinical tests, and trials.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Commercial Kits

RevertAid First Strand cDNA Synthesis Kit (K1622, Thermo Scientific) and Pierce BCA Protein Assay Kit (23227, Thermo Scientific) were used in this study.

3.1.2. Chemicals and Reagents

In this study following chemicals and reagents were used: Ethanol (E/0600DF/17, Fisher Scientific), Methanol (M/4050/15, Fisher Scientific), 2-Propanol (P/7507/17, Fisher Scientific), Chloroform (C/4960/17, , Fisher Scientific), 30% Acrylamide/Bis-acrylamide (A2-0064, Geneflow), Hi-Res Standard Agarose (AGD1, Geneflow), 0.5M EDTA pH 8.0 (15575-038, Invitrogen), Immobilon Forte Western HRP substrate (WBLUF0100, Merck), 10X Tris/Glycine SDS Running Buffer (B9-0032, Geneflow), 10X Tris Glycine Transfer Buffer (B9-0056, Geneflow), Skimmed milk (non-fat powder, SKI400, Biochop), TRIS (AC228030051, Fisher Scientific), APS (A3678, Sigma), BSA (A3059, Sigma), Qiazol reagent (Qiagen, 79306), Nitrocellulose blotting membrane (10600002, Amersham), Tween-20 (P1379, Sigma-Aldrich), TEMED (EC-503, National Diagnostics), 2-Mercaptoethanol (M3148, Sigma), Glacial Acetic Acid (BP1185, Fisher), Sodium Dodecyl Sulfate (S/5200/53, Fisher Scientific), Dimethyl Sulfoxide (D5879, Sigma Aldrich), Glycine (G45, Fisher Scientific), Sodium Chloride (S271, Fisher Scientific), Sodium Hydroxide (106498, Merck), Hydrochloric acid (H/1200/PB15, Fisher Scientific), Glycerol (C5516, Sigma) Bromophenol Blue (BP115, Fisher Scientific), GelGreen Nucleic Acid Stain 10000X (SCT125, Merck), Crystal Violet (C-3886, Sigma), GeneRuler DNA Ladder Mix, ready-to-use (SM0333, Thermo Scientific), Precision Plus Protein All Blue Prestained Protein Standards (1610373, Bio-Rad), Oxaliplatin (Hospira UK), Doxorubicin (D1515, Sigma), TRAIL (310-04, Peprotech), zVAD-FMK (627610, Merck), MG-132 (474787, Merck) (Bortezomib 5043140001, Merck), TMRE (T669, Invitrogen), RNase A (556746,

Sigma-Aldrich), Propidium Iodide (P4864, Sigma-Aldrich), MyTaq Red Mix (BIO-25043, Meridian Bioscience).

3.1.3. Buffers and Solutions

Buffers and solutions used in this study are given in **Table 3.1** and SDS-PAGE gels were prepared using the recipes in **Table 3.2** and **Table 3.3**.

Table 3.1: Recipe of buffers and solutions.

Buffer	Components
50X TAE buffer	242 g Tris-base 57.1 ml glacial acetic acid 100 ml 0.5M EDTA (pH 8.0) Adjust volume to 1L with distilled H_2O (dH_2O)
10X TBS	100 mM Tris (pH 8.0) 1.5 M NaCl Adjust volume to 1L with dH_2O
1X TBS-T	100 ml 10X TBS 900 ml dH_2O 0.1% Tween-20
2X Laemmli Buffer	23.75 ml dH_20 6.25 ml 0.5 M Tris (pH 6.8) 5 mL Glycerol 10 mL 10% SDS Dilute desired amount using 2:5 ratio with dH_2O to obtain 2X Laemmli buffer
5 X SDS-PAGE Gel Loading Buffer	3.8 ml dH_20 1 ml 0.5 M Tris (pH 6.8) 0.8 ml Glycerol 1.6 ml 10% SDS Add freshly following components: 0.4 ml 2-mercaptoethanol 0.4 ml 0.05% Bromophenol blue (BPB)
Crystal Violet Staining Solution (0.5%)	0.5 g crystal violet powder 20 ml methanol Adjust volume to 100 ml dH_2O
1% agarose	1 g agarose 100 ml 1X TAE buffer
2.5% Primary Antibody Dilution Buffer	1.25 g BSA Adjust volume to 50 ml 1X TBS-T
5% Blocking and Secondary Antibody Dilution Buffer	2.5 g skimmed milk powder Adjust volume to 50 ml 1X TBS-T
Cell freezing media	10% DMSO 90% FBS

Table 3.2: 5% stacking gel recipe per gel casting mold.

5% Stacking Gel	**4 ml**
dH_2O	2.7
30% Acrylamide/Bis-acrylamide	0.67
1 M Tris (pH 6.8)	0.5
10% SDS	0.04
10% APS	0.04
TEMED	0.004

Table 3.3: Resolving gel recipe in different concentrations per gel casting mold.

Resolving Gel (10 ml)	**6%**	**8%**	**10%**	**12%**	**15%**
dH_2O	5.3	4.6	4	3.3	2.3
30% Acrylamide/Bis-acrylamide	2	2.7	3.3	4	5
1.5 M Tris (pH 8.8)	2.5	2.5	2.5	2.5	2.5
10% SDS	0.1	0.1	0.1	0.1	0.1
10% APS	0.1	0.1	0.1	0.1	0.1
TEMED	0.008	0.006	0.04	0.04	0.04

3.1.4. Cell Lines and Cell Culture Reagents

DLD1, HCT116, SW480, MDA231 and SNU387 cells were obtained from American Type Culture Collection (ATCC; Rockville, MD). Dulbecco's Modified Eagle's Medium (DMEM) (D5671, Sigma), Fetal Bovine Serum, Heat Inactivated (FBS) (EU-000-F, Sera Laboratories), L-Glutamine (G7513, Sigma), 100X Penicillin-Streptomycin (P4333, Sigma), 1X Trypsin-EDTA Solution (T3924, Sigma), 10X PBS (phosphate buffered saline) pH 7.4 (10010023, Gibco) were used for cell maintenance.

3.1.5. Oligonucleotides

Primers in **Table 3.4** were obtained from Sigma Aldrich.

Table 3.4: Forward and reverse primers used in gene expression studies.

Gene	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
PARP1	TTTGGGCAAAACTACCCCTG	TACCCATCAGCAACTTAGCGG
ERCC1	CTCCCGGGTGACTGAATGTC	TTCAGAGTCTGGGGAGGAGG
ERCC4	CATCCATCCGCTTCTGGGTT	GACATGGAGATGCACTGGCT
GAPDH	GGCTGAGAACGGGAAGCTTGTCAT	CAGCCTTCTCCATGGTGGTGAAGA

3.1.6. Antibodies

All primary and secondary antibodies used in this study were listed in **Table 3.5**.

Table 3.5: List of antibodies used in the study.

Antibody	Company	Catalog No.	Dilution
PARP-1	Cell Signaling	9542S	1:1000
p-p53	Cell Signaling	9286P	1:1000
p53	Santa Cruz	SC-6243	1:300
p-CHK1	Cell Signaling	2348P	1:500
p-CHK2	Genetex	GTX132204	1:500
p-H2A.X	Cell Signaling	2577S	1:3000
H2A.X	R&D Systems	MAB3406	1:300
β-Actin	BD Biosciences	612656	1:3000
ERCC1	Cell Signaling	12345S	1:1000
ERCC2(XPD)	Abclonal	A19241	1:500
ERCC3(XPB)	Abclonal	A12702	1:500
ERCC4(XPF)	Cell Signaling	13465S	1:500
ERCC5(XPG)	Genetex	GTX110609	1:500
XPA	R&D Systems	AF3416	1:500
XPC	Abclonal	A8354	1:500
DDB1	Elabscience	E-AB-12364	1:500
DDB2	Abclonal	A11615	1:1000
Anti-Mouse HRP	Dako	P016102-2	1:3000
Anti-Rabbit HRP	Dako	P021702-2	1:3000
Anti-Goat HRP	Santa Cruz	SC-2020	1:3000

3.1.7. Equipment

Laminar flow cabinet, Class II (PMV Clean Air), Refrigerated Laboratory Centrifuge (Heraeus Instruments Function Line), Refrigerated Laboratory Minicentrifuge (Beckman Coulter microfuge 22R), Freezer, -80ºC (ESCO), CO_2 Incubator (Labogene, Benchtop 6400-4), Vertical Electrophoresis Cell for SDS-PAGE (BioRad Mini-PROTEAN Tetra), Horizontal Mini Gel System (BioRad), Inverted Light microscope (Nikon), Water Bath (Lauda AL12), High precision laboratory scale, 0,1 mg (Sartorius BL210S), Electronic pipette controller (PipetAid, Biohit Midi Plus), Freezer, -20 ºC (Arcelik), Heat-block (Denville Scientific), Ultrapure water purification system (Merck Millipore) Refrigerator (No-frost, Arcelik), Ice maker (Brema ice maker), Magnetic stirrer with hot plate (Denville Scientific), Liquid

Nitrogen Storage Vessel (Arpege 140), Ultrasonic homogenizer (Branson), Microplate Reader (Varioskan Flash, Thermo Scientific), NanoDrop 8000 Spectrophotometer (Thermo Scientific), Microwave (Kumtel), Orbital Shaker (Denville Scientific), Thermo Cycler (Biometra), Electrophoresis power supply (BioRad), Gel/Blot Imaging Systems (Biorad ChemiDOC XRS and Amersham Image Quant 800), UV-Transilluminator (Vilber Lourmat), Qubit 3 Flourometer (Thermo Fisher) Autoclave (Nüve), Flow Cytometer (BD FACSCalibur).

3.2. Methods

3.2.1. Cell Culture

3.2.1.1. Maintenance of Cell Lines

DLD1, HCT116, SW480, MDA231 and SNU387 cell lines were maintained in a humidified incubator at 37°C under 5% CO2. Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 2mM L-Glutamine and 1% of 100X Penicillin-Streptomycin was used as growth media for all cell lines.

To defrost the cells, vials were transferred from liquid nitrogen tank (-196) to ice and then were thawed rapidly in a 37^{o}C water bath. Cells were diluted with warm DMEM and centrifuged for 5 min at 1500 rpm to remove DMSO. Then cell pellets were re-suspended with culture media and transferred to the appropriate culture flasks to obtain maximum recovery.

For cell propagation, cells were washed with 1X PBS, then 1X Trypsin-EDTA was added, and cells were incubated for 5 min at 37^{o}C allowing the adherent cells to detach. As a last step, cells were re-suspended in growth media at the appropriate concentration and transferred in a new flask or seeded in wells/dishes for experiments. Cell growth was monitored using a light microscope and the confluency of the cells was maintained around 80-90% during propagation.

Cell counting was performed using a hemocytometer chamber. Cells within 25 squares in the center were counted under a light microscope. 20µl from the final dilution placed onto both side of dual chamber. The final cell count was calculated using the formula below.

10^4 (constant number) x Amount of counted cell in 25 squares = Cell number / ml

Total cell number = Cell number / ml x Total volume of cells

For freezing cells, cells were detached by trypsinization and centrifuged 1500 rpm for 5 min. The supernatant was discarded, and pellet was re-suspended with cold freezing media composed of 10% DMSO and 90% FBS. Then they were distributed to cryo-vials and placed in a -80ºC freezer first and 24h later vials were transferred to liquid nitrogen for long-term storage.

3.2.1.2. Cell Treatments

Cells were treated with two different DNA-damaging agents, oxaliplatin or doxorubicin in different concentrations or TRAIL to induce apoptosis and zVAD-fmk, a caspase inhibitor, was used to restore the cell survival. For proteasome-dependent degradation studies, cells were treated with MG132 or Bortezomib to inhibit proteasomal activity.

10μM zVAD-fmk was used for caspase inhibition experiments and cells were pre-incubated with zVAD-fmk for 30 min before adding DNA-damaging agents. The specific incubation times/time points and/or dosage of oxaliplatin, doxorubicin and MG132 were indicated in the related experiment.

3.2.2. Total Protein Isolation

Cells were collected by spinning down at 1500 rpm for 5 min and washed in 1 x PBS and pelleted by spinning down at 4000 rpm for 2 min. According to pellet size, 2X Laemmli buffer was added, and samples were sonicated via a sonicator with a probe for 30 sec at 1.5Hz.

3.2.3. Bicinchoninic Acid (BCA) Assay

The total protein amount in cell lysates was measured by using the Pierce BCA Protein Assay Kit according to the manufacturer's protocol. Absorbance was measured at 562 nm. By using diluted BSA standards, a standard curve was generated, and protein concentrations were determined via a formula generated from the standard curve.

3.2.4. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is a technique to separate proteins on a gel according to their size by creating an electrical field. In this study, different separating gel concentrations were used. Prepared gel was poured between glass plates and left for polymerization. After gel was polymerized, 5X Laemmli buffer including freshly added 2-Mercaptoethanol was added to 20 μg of each protein samples and samples were boiled at 95 ^{0}C for 10 min to enable better denaturation. Then samples were loaded in equal concentration into wells and were run in 1X Tris/Glycine SDS Running Buffer at 120V for 20 min first and then at 180V for another 50-60 min.

3.2.5. Transfer and Immunoblotting (Western Blotting)

Once running finished, proteins were transferred to nitrocellulose membrane by a technique called wet transfer in 1X Tris-Glycine Transfer Buffer including 20% methanol. The transfer process was performed at 22V for overnight at 4ºC. After transfer, the membrane was blocked in 5% blocking solution for 30 min at room temperature. Then membrane was treated with primary antibody diluted in 2.5% BSA in TBS-T solution and incubated for 30 min at room temperature. The membrane was washed with TBS-T 3 times for 15 min in total. The membrane was then incubated with a secondary antibody conjugated with horseradish peroxide (HRP) diluted in 5% skimmed milk solution for another 30 min at room temperature and was washed 3 times for 15 min in total. All incubations and washes were performed on an orbital shaker.

3.2.6. Imaging

Immobilon Forte Western HRP substrate was used to image the bands according to manufacturer's instructions. The chemiluminescent signals were captured by using two different imagers (Biorad ChemiDOC XRS and Amersham Image Quant 800). Band intensity was quantified by using ImageJ.

3.2.7. Total RNA Isolation

Total RNA was extracted by using Qiazol reagent according to following instructions.

1. Cells were collected by spinning down at 1500 rpm for 5 min and washed in 1 x PBS and pelleted by spinning down at 4000 rpm for 2 min.
2. 700ml Qiazol reagent was added to pellets, and they were resuspended by pipetting.
3. Samples incubated for 5 min at room temperature.
4. 200ml chloroform was added, and samples were mixed by inverting and incubated for further 3min and mixed again by inverting before centrifuge.
5. Samples were centrifuged at 12000xg for 15min at 4°C.
6. The upper, aqueous phase was transferred to a new tube and 350ml isopropanol was added and mixed by inverting.
7. Samples were incubated for overnight at -20°C.
8. Samples were centrifuged at 12000xg for 15min at 4°C.
9. Supernatant was discarded and 700ml of 90% ethanol was added to samples without disturbing RNA pellets.
10. Samples were incubated for overnight at -20°C.
11. Samples were centrifuged at 12000xg for 15min at 4°C.
12. Supernatant was removed completely, and RNA pellets were air-dried.
13. RNA pellets were dissolved in an appropriate volume of RNase-free water.

The quality and quantity of RNA samples were determined by NanoDrop 8000 Spectrophotometer. Obtained RNA samples were used straightaway to synthesize cDNA or stored at -80°C.

3.2.8. cDNA Synthesis

RevertAid First Strand cDNA Synthesis Kit was used to convert total RNA into cDNA using oligo(dT) according to manufacturer's instructions. Total volume of reaction mixture was 20 µl and obtained cDNA was then diluted 1/3 and either used immediately for next step or stored at -20°C.

3.2.9. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR reaction was performed using MyTaq Red Mix with the primers shown in section 3.1.5 (**Table 3.4)**. 2µl of cDNA was used as a template. PCR was set up according to components and cycling conditions shown in **Table 3.6** and **Table 3.7**.

Table 3.6: PCR mixture used for the reaction.

Component	Amount
MyTaq Red Mix, 2x	10µL
Forward primer	1µL
Reverse primer	1µL
cDNA	2µL
Ultra-pure water	Up to 20µL

Table 3.7: PCR cycling conditions.

Initial Denaturation	94ºC	5 min	
Denaturation	94ºC	25 sec	X 26-31 cycles
Annealing	60ºC	25 sec	
Extension	72ºC	25 min	
Final Extension	72ºC	5 min	

3.2.10. Agarose Gel Electrophoresis

1% agarose gels were used for the observation of the PCR products. 1 g agarose was dissolved in 100 ml of 1X TAE buffer and 3 ml of GelGreen Nucleic Acid Stain was added to solution. GeneRuler DNA Ladder Mix was used to evaluate the PCR products size. 7µL of each sample were loaded. The gels were run for an average of 25-30 minutes in 100V in 1X TAE buffer. The results were monitored via UV-Transilluminator.

3.2.11. Apoptosis Assay

Cells were seeded into wells and treated with oxaliplatin, doxorubicin or TRAIL to induce apoptosis using different concentrations and different incubation times. All cells, including floating ones, were collected after completion of treatments. Ten or twenty percent of the cells was used for either sub-G1 analysis in doxorubicin-treated cells using PI staining or mitochondrial membrane potential assay in TRAIL- or

oxaliplatin-treated cells using TMRE. The rest of the cells were used to extract proteins for western blotting to observe PARP cleavage. Unless otherwise indicated, 6h and 16h incubation time performed for TRAIL and DNA damaging agents, respectively.

TMRE (Tetramethylrhodamine, ethyl ester) is a cell permeant dye with red-orange fluorescent which accumulates in active mitochondria so that it is used to determine mitochondria depolarization ($\Delta\Psi m$) via flow cytometry. Due to doxorubicin's intrinsic florescence property, an alternative method, e.g., propidium iodide (PI) staining was performed to analyze DNA fragmentation (Sub-G1) in experiments involving doxorubicin. After collection of the cells, they were re-suspended in PBS and 100µl ($1/5^{th}$ of sample) of cell suspension was reserved for TMRE assay prior to pelleting for cell lysis. TMRE stock solution (100 µM) was diluted in 1:1000 ratio in growth media and 400µl of the prepared solution was added to each sample and cell were incubated at 37°C for 30 min.

For PI staining, 70% ethanol was added into 100µl of cell suspension in PBS and fixed for overnight at -20°C. Following day, fixed cells were centrifuged to remove the ethanol and re-suspend again in 150 µL PBS. Afterwards, 200 µL RNase (0.260 Kunitz unit) and 400 µL PI (50 µg/ml) was added to each sample, respectively and cells were incubated at 37°C for 30 min. TMRE assay and PI staining were analyzed using FACSCalibur flow cytometer.

3.2.12. Colony Formation Assay

Cells seeded at low density (2000 cells/6 cm dish) were treated with 1, 10 or 100 nM BTZ for 6 h and then 1µM oxaliplatin was added to cells. Once colonies formed (after 14 days), cells were washed, fixed with 95% ethanol, and stained with crystal violet for counting.

3.2.13. Statistical Analysis

Graphics represent the mean ± SEM, unless otherwise stated. The differences in mean-relative values were tested by two-tailed paired student t-test using GraphPad Prism 9 software. The significance threshold was set at 0.05 for all statistical tests. The

standard error of mean is presented as “±”. Each experiment was repeated at least 3 times.

4. RESULTS

4.1. Abundance of NER Components upon Treatment with Different Concentrations of DNA-Damaging Agents

To examine the abundance of all NER components upon DNA damage, SW480 cells were treated with 2 different DNA-damaging agents, oxaliplatin and doxorubicin in low and high concentrations. We observed that protein abundance and modifications happen when DNA damage response pathway is activated by showing increased phosphorylation of H2aX, p53, CHK1 and CHK2. The total abundance of H2aX protein remained unchanged. PARP was cleaved after treatment in both concentrations of the two drugs (**Figure 4.1A**) indicating initiation of apoptosis.

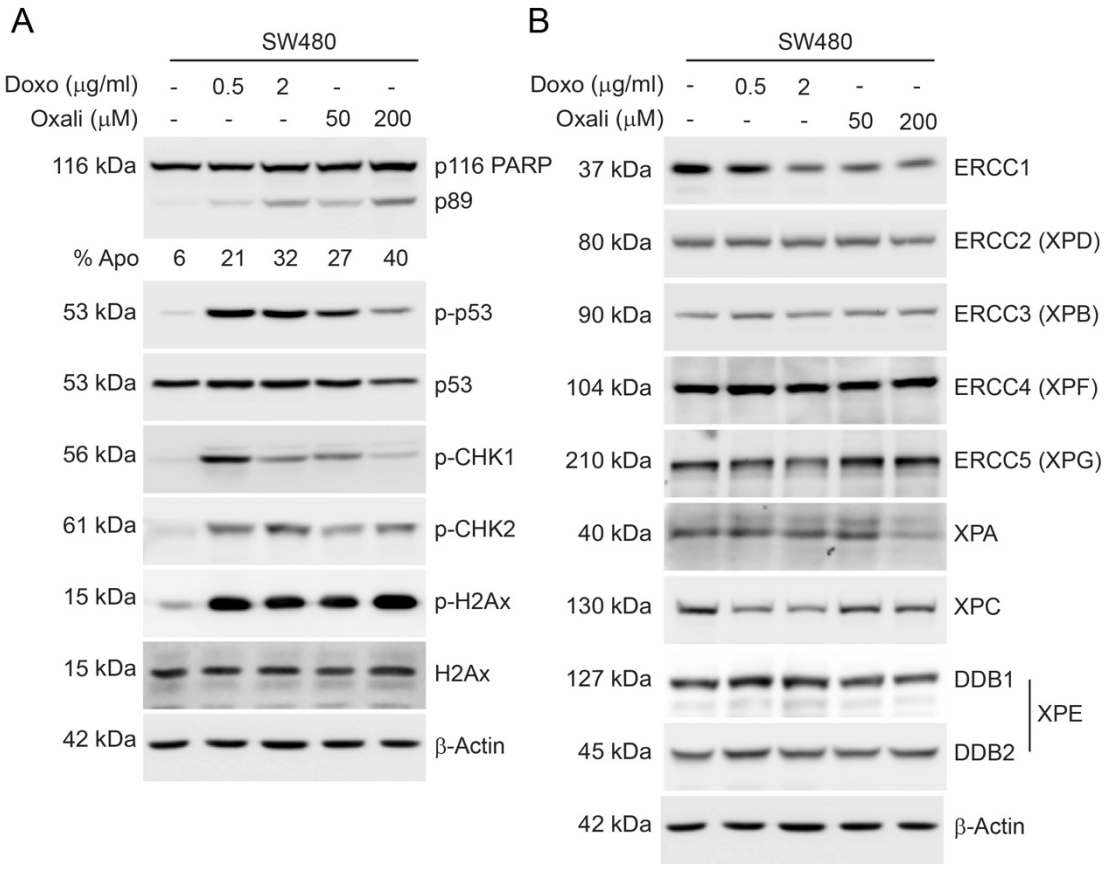

Figure 4.1: NER components upon treatment with DNA-damaging agents. SW480 cells were treated with doxorubicin (0.5- and 2μg/ml) or oxaliplatin (50- and 200μM).

We detected no change in DDB1, DDB2, XPD, XPB, XPF and XPG but ERCC1 level dramatically decreased. XPA decreased in only the sample treated with high concentration of oxaliplatin. Finally, reduced XPC abundance was observed in only Doxorubicin-treated cells (**Figure 4.1B**). DNA fragmentation was also measured by flow cytometry using PI staining (**Figure 4.2**) to quantitatively analyze apoptosis.

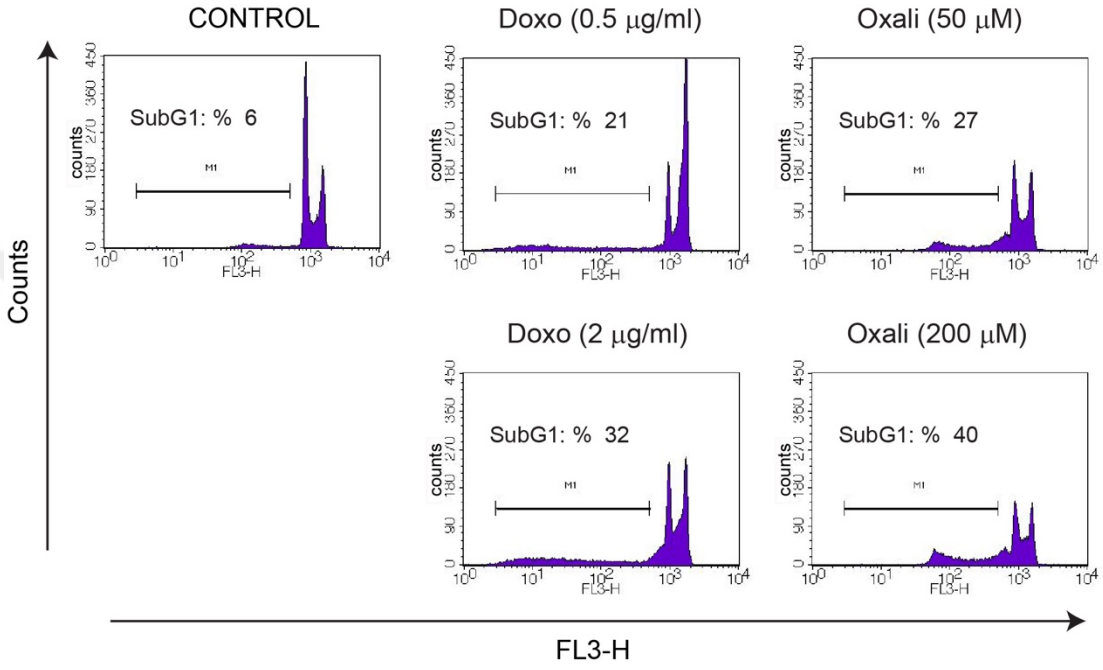

Figure 4.2: Genotoxic drug-induced DNA fragmentation analysis. SW480 cells were treated with Doxorubicin (0.5-2μg/ml) or oxaliplatin (50-200μM) for 16h and subjected to propidium iodide staining. Presence of DNA with staining intensity less than the G1 peak, indicated with SubG1, registers as apoptosis and displayed in individual panels.

4.2. The Contribution of Caspase Inhibition to the Decrease of NER Component Proteins

To examine the possible involvement of caspases in the reduction of ERCC1, XPC and XPA proteins, SW480 cells were treated with oxaliplatin (200μM) or doxorubicin (2μg/ml) with or without pan-caspase inhibitor zVAD-fmk. Our results showed that we efficiently inhibited caspases using zVAD-fmk, as indicated by the absence of cleaved PARP (p89) (**Figure 4.3**). In line with previous observations, total- and phospho-p53 protein abundance also recovered by the caspase inhibitor in oxaliplatin-treated sample [109]. XPA protein level was restored with zVAD-fmk suggesting it is potentially a caspase target. ERCC1 abundance remained low in both DNA damaging

treatments, regardless of zVAD-fmk treatment, suggesting that ERCC1 is not cleaved by caspases and consistent in samples where apoptosis (DNA fragmentation) is more than 25%. The decrease of XPC protein in doxorubicin treated sample also was not restored with caspase inhibition suggesting it is a doxorubicin treatment-related post-translational event resulting in decreased protein levels. XPF, XPG and DDB1 protein levels were also analyzed as controls in this assay showed no change (**Figure 4.3**).

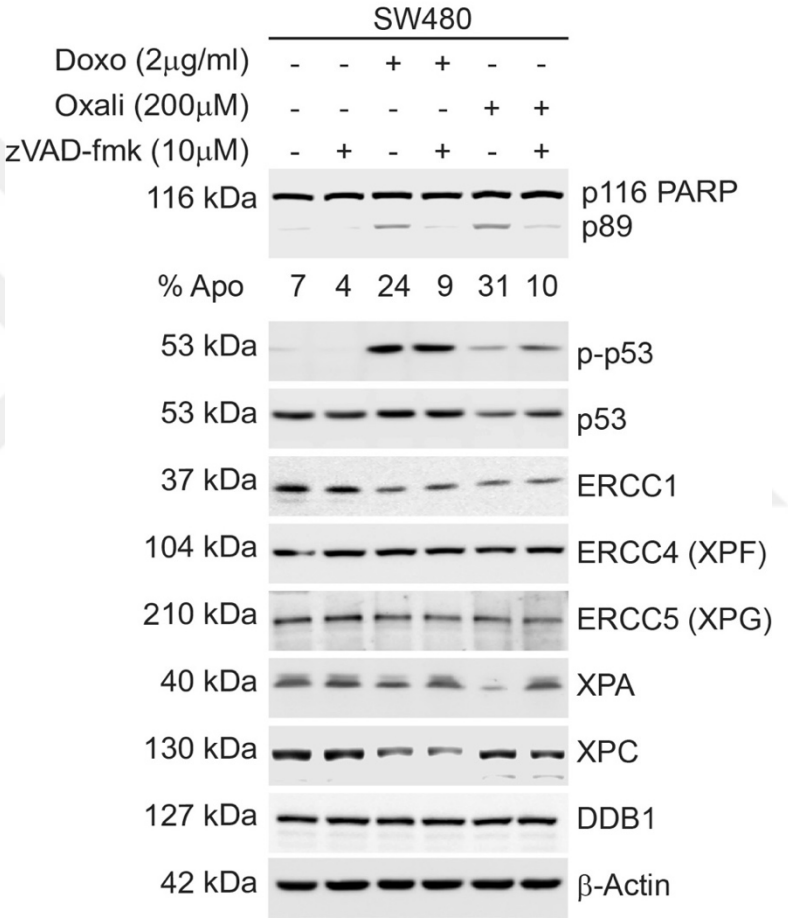

Figure 4.3: Effect of caspase inhibitor on NER components in doxorubicin- or oxaliplatin-treated SW480 cells. Cells were pre-incubated 10μM zVAD-fmk for 30min and then further incubated with doxorubicin (2μg/ml) or oxaliplatin (200μM) for 16h.

DNA fragmentation was also measured by flow cytometry using PI staining (**Figure 4.4**). These results suggest ERCC1, XPA and XPC as potential proteins regulated during apoptosis. Hence, ERCC1 is the prime target to be further investigated in DDR (apoptosis-DNA repair link), particularly in oxaliplatin-induced apoptosis.

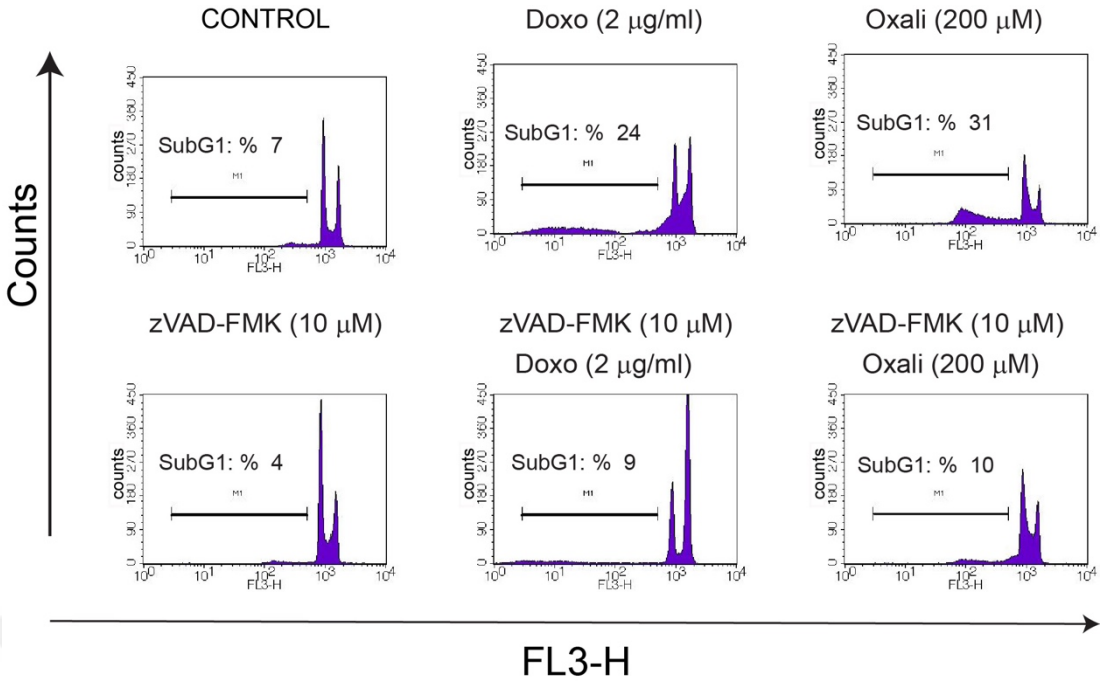

Figure 4.4: SubG1 DNA analysis of SW480 cells treated with caspase inhibitor and genotoxic agents. SW480 cells were pre-treated with caspase inhibitor zVAD-fmk for 30 mins and incubated 16h with doxorubicin (2μg/ml) or oxaliplatin (200μM). SubG1 analysis of was performed as indicated in previous panel. zVAD-fmk effectively inhibits effector caspase activation and DNA fragmentation.

4.3. The Impact of DNA Damage-Induced or Death Receptor-Mediated Apoptosis to ERCC1 Abundance

To find out if the decrease in ERCC1 protein level was a DNA damage-specific event, cells were treated with oxaliplatin, a bulky adduct-forming chemotherapeutic, doxorubicin, a topoisomerase II inhibitor and double-strand break-inducing agent or TRAIL, a death receptor ligand, and the changes in ERCC1 levels were observed using western blotting. To that end, cells were treated with 200μM oxaliplatin or 2μg/ml doxorubicin for 16h or 250 ng/ml TRAIL for 6h, lysates prepared, and western blotting performed for ERCC1, PARP (marker of caspase activation, therefore apoptosis) and actin (equal loading control). To generalize our findings, we used three different colorectal (DLD1, HCT116 and SW480), one breast (MDA231) and one hepatocellular carcinoma (SNU387) origin cancer cell lines. Mitochondrial membrane potential and DNA fragmentation were assessed by using TMRE assay and propidium iodide (PI) staining, respectively to quantitively analyze apoptosis. We could not use

TMRE for doxorubicin-treated cells so that we used PI staining as an indicated of late apoptosis. TRAIL-treated cells were used as positive control for PI staining. Our initial results indicated a decrease of ERCC1 protein upon induction of DNA damage-induced apoptosis at an early stage that PARP cleavage is barely detectable in all cells which were treated with oxaliplatin or doxorubicin (**Figure 4.5A-B**).

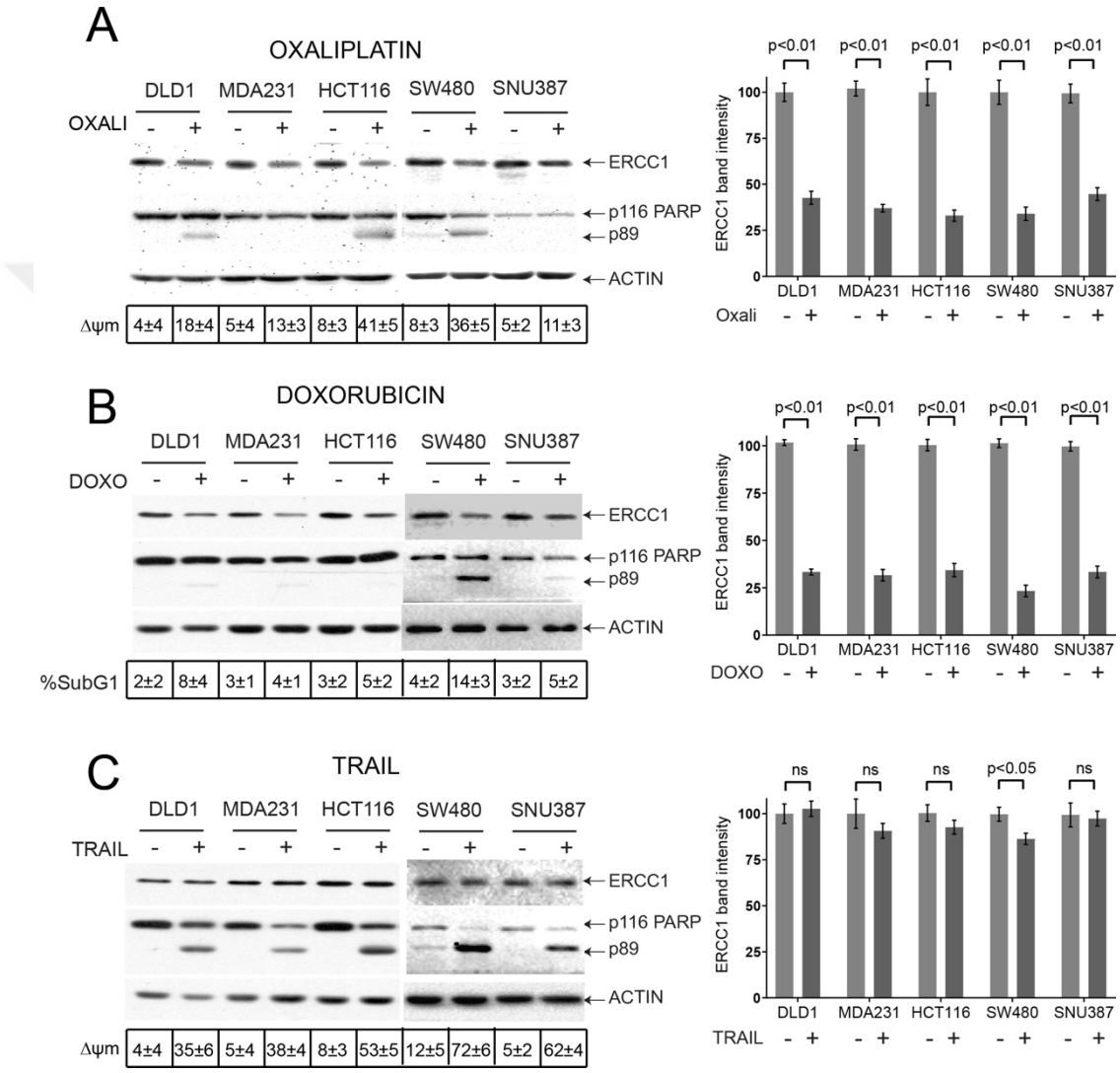

Figure 4.5: ERCC1 abundance upon treatment with DNA damage-inducing agents or a death receptor ligand. Five carcinoma cell lines were treated with (A) oxaliplatin (200μM) or (B) doxorubicin (2μg/ml) or (C) TRAIL. ERCC1 band intensities were presented on the right side of western blot images. Percent of apoptosis was presented under each lane of western blotting images.

On the other hand, TRAIL, which activates extrinsic apoptosis pathways, caused more apoptosis than the treatments with DNA-damaging agents as assessed by extensive

PARP cleavage and mitochondria depolarization (by using TMRE staining and flow cytometry). However, no decrease was observed in ERCC1 levels in treated samples as compared to controls (**Figure 4.5C**). In addition to PARP cleavage, our TMRE (ΔΨm) and PI (SubG1%) results confirmed the apoptosis in TRAIL-, oxaliplatin- and doxorubicin-treated cells (**Figure 4.6** and **Figure 4.7**). Our overall results suggest that the observed decrease in ERCC1 abundance in oxaliplatin and doxorubicin treatments can be associated with DNA damage recognition, repair, and apoptosis.

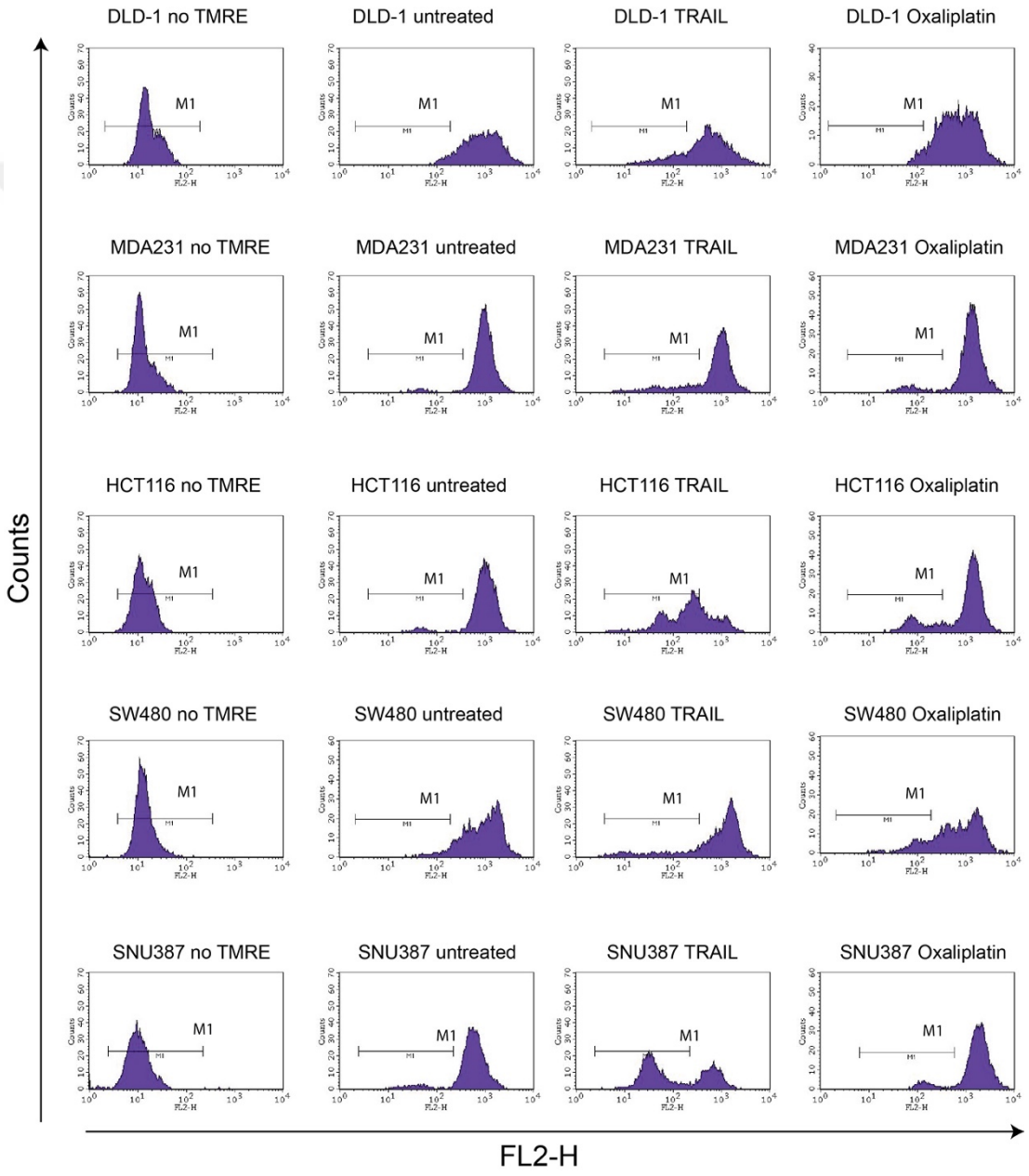

Figure 4.6: Flow cytometry results for TRAIL- or oxaliplatin-treated cell lines. After drug treatments, cells were incubated with TMRE (100nM) for 30min for flow cytometry analysis.

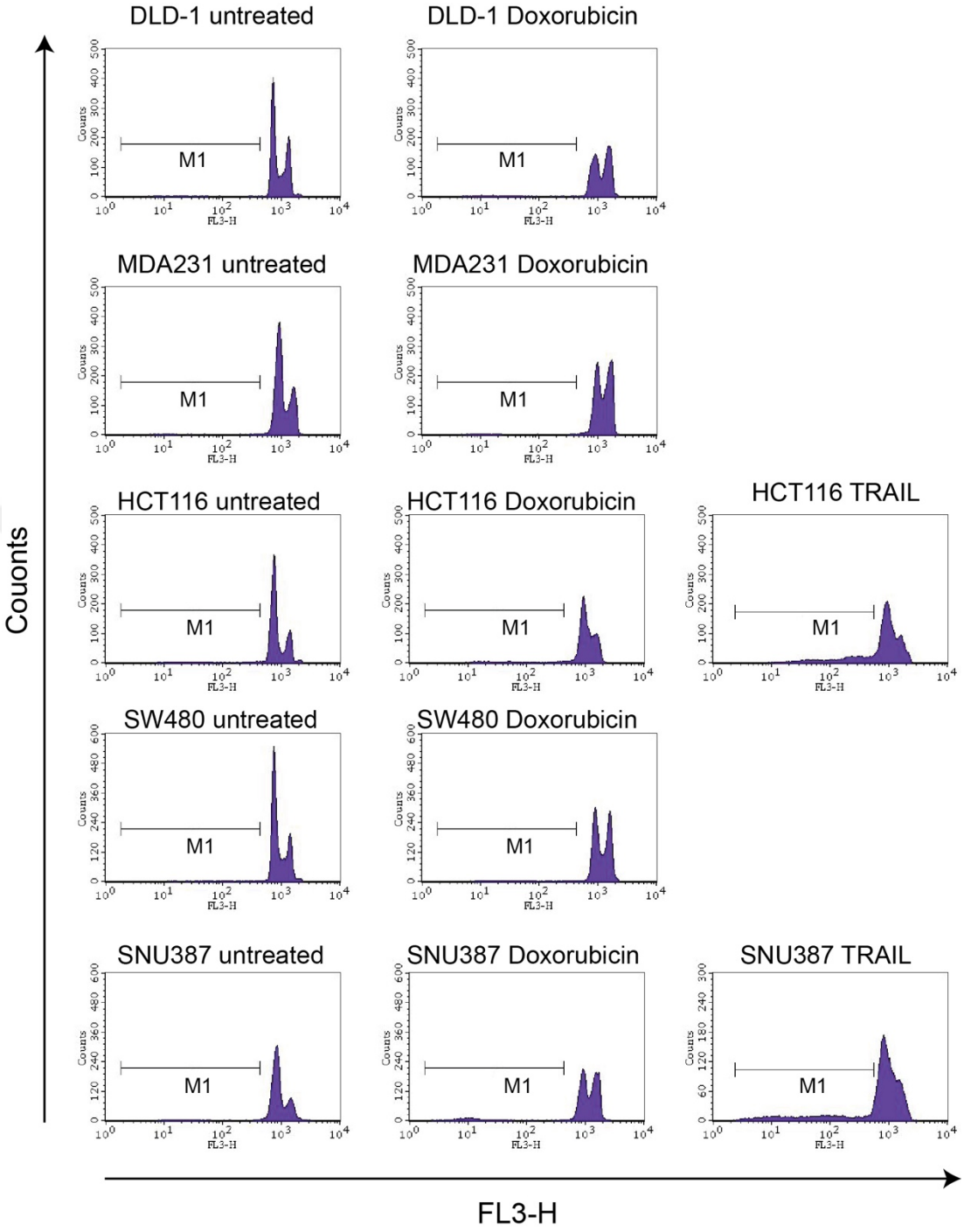

Figure 4.7: Flow cytometry results for doxorubicin-treated cell lines. After drug treatment, cells were fixed with 70% EtOH for overnight and then cells were further incubated with 0,260 Kunitz unit RNase A and 50 µg/ml PI for 30 min.

4.4. Kinetics/Dose Response Analysis of ERCC1 Abundance upon Treatment with DNA-Damaging Agents

In order to have a better understanding between the temporal relationship of apoptosis initiation/progression and decreased ERCC1 levels, kinetics and concentration curve experiments were carried out using DNA damaging agents. We selected 2 cell lines (DLD1 and SW480) from our panel to further investigate the decrease of ERCC1 in a time and concentration manner. We initially treated cells with oxaliplatin and collected samples at fixed time points using a medium dose of the agents or upon treatment with different concentrations of the agents at fixed time. We observed the appearance of cleaved PARP and the decrease in ERCC1 levels coincide suggesting and supporting our previous results that ERCC1 decrase is aligned with the initiation of apoptosis. Importantly ERCC1 decrease was visible in SW480 cells before significant PARP cleave was detected, suggesting it may be happening before caspase activation (**Figure 4.8**).

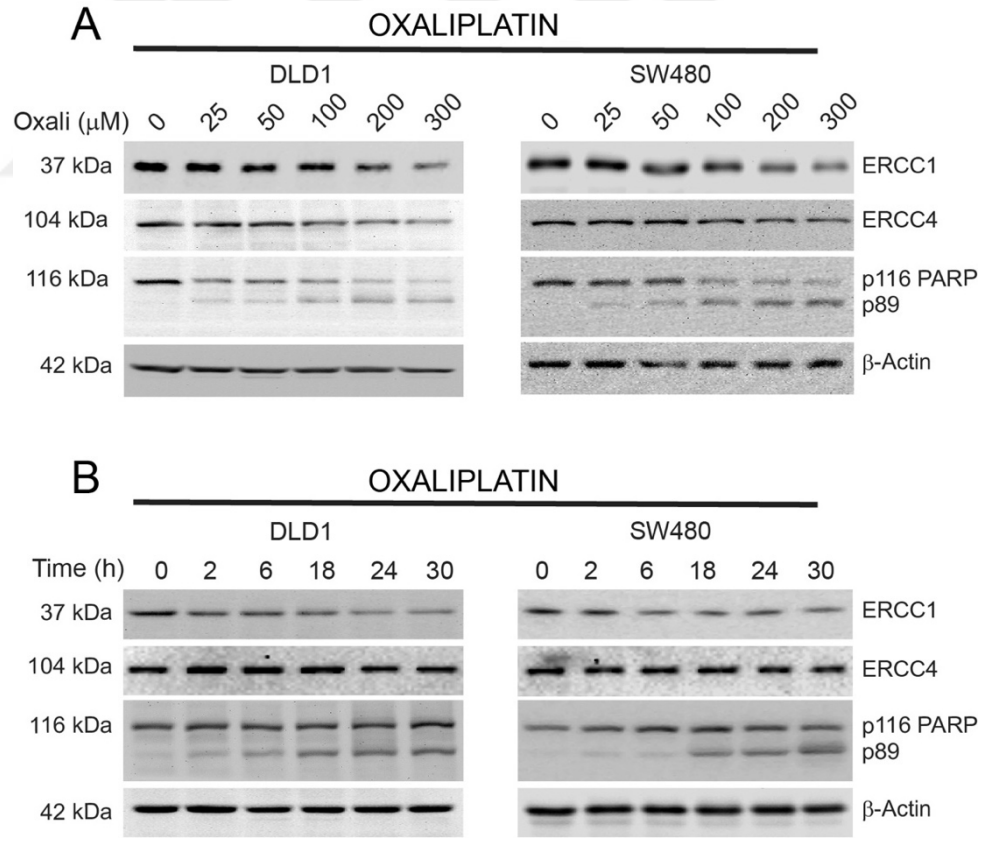

Figure 4.8: Kinetics/dose response analysis of ERCC1 upon oxaliplatin treatment of DLD1 and SW480 cell lines. (A) Cells were incubated with oxaliplatin in different concentrations at fixed time (16h). (B) Cells were incubated with same oxaliplatin concentration (200 μM) and collected at different time points.

The experimental and assessment conditions were repeated for doxorubicin treatment. Doxorubicin induced cell death was similarly pronounced, yielding PARP cleavage and ERCC1 decrease (**Figure 4.9**) suggesting its downregulation is a very early event during apoptosis. In these experiments we also use ERCC4, another component of NER, as an internal positive control and no concernable change in ERCC4 protein level was detected (**Figure 4.8** and **Figure 4.9**). We concluded earlier time points (e.g. 1 or 2h) and lower doses of oxaliplatin (e.g. 50-100μM) should be considered to map the precise timing of ERCC1 decrease in relation to mitochondira depolarization, which is the point of no return in intrinsic apoptosis pathway.

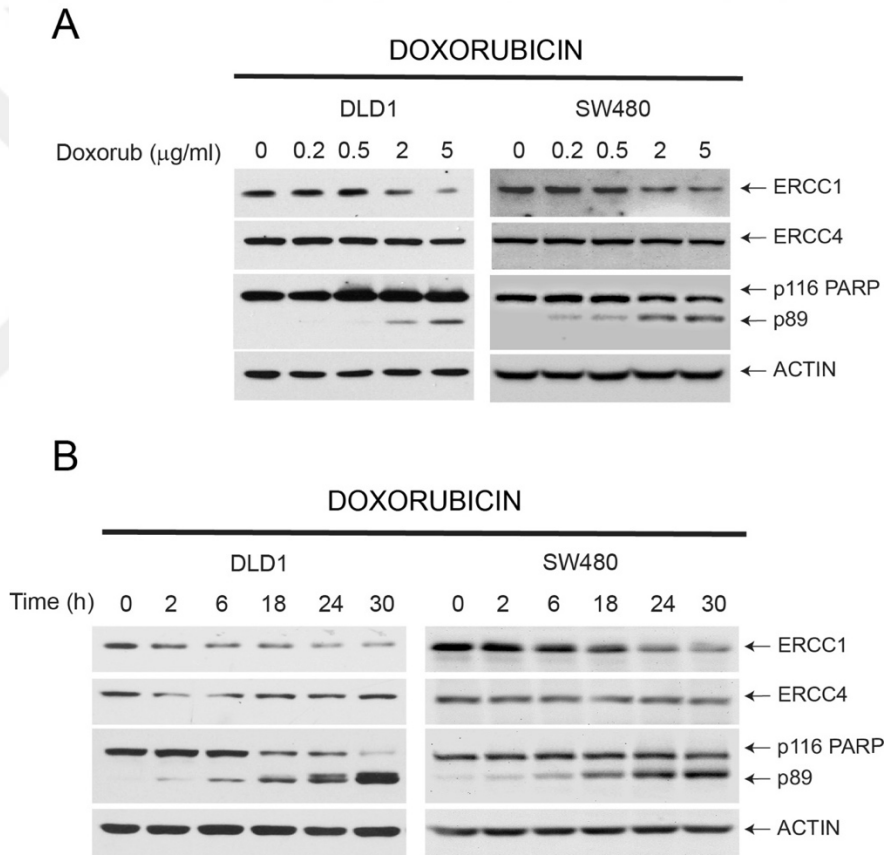

Figure 4.9: Kinetics/dose response analysis of ERCC1 upon doxorubicin treatment of DLD1 and SW480 cell lines. (A) Cells were incubated with different doxorubicin concentrations at fixed time (16h). (B) Cells were incubated with the same doxorubicin concentration (2μg/ml) and collected at different time points.

4.5. Detailed Kinetic Analysis of ERCC1 Gene Expression and Protein Changes upon Oxaliplatin Treatment

In previous experiments, we showed that ERCC1 protein abundance decreases upon DNA damage by using western blotting. The observed decrease could be a result of changes in transcriptional, translational, or post-translational regulations. It is also to find out whether that the change in ERCC1 levels is pre- or post-mitochondrial event. To find out whether ERCC1 transcription or translation is changed in that process and to determine precise timing of ERCC1 downregulation, we performed a kinetics experiment where we treated SW480 cells with oxaliplatin (100μM) for up to 16h and collected cells at early and late time points for assessing RNA and protein abundance of ERCC1 by semi-quantitative PCR and western blotting, respectively. Our results showed that both *ERCC1* and *ERCC4* mRNA expression sharply decreases at 1h after oxaliplatin treatment, and then recovering to pre-treatment levels as of 4h time point. In this experiment, we also analyzed *PARP1* gene as a member of DNA damage repair-apoptosis pathway and *GAPDH* gene as an equal loading control. However, we did not see any change in their gene expression during treatment (**Figure 4.10A-B**).

Although the gene expression of *ERCC1* and *ERCC4* showed a similar trend, their protein abundance did not change in a similar manner. In line with our previous observations, there was no change observed in ERCC4 protein level but ERCC1 protein remained constant up to 2h and then decreased at 4h and onwards (**Figure 4.11**). These results indicate a unique control mechanism where ERCC1 protein is decreasing, independent of its transcription, in cells that are committed to die via apoptosis as observed at much later point, 16h, by PARP cleavage.

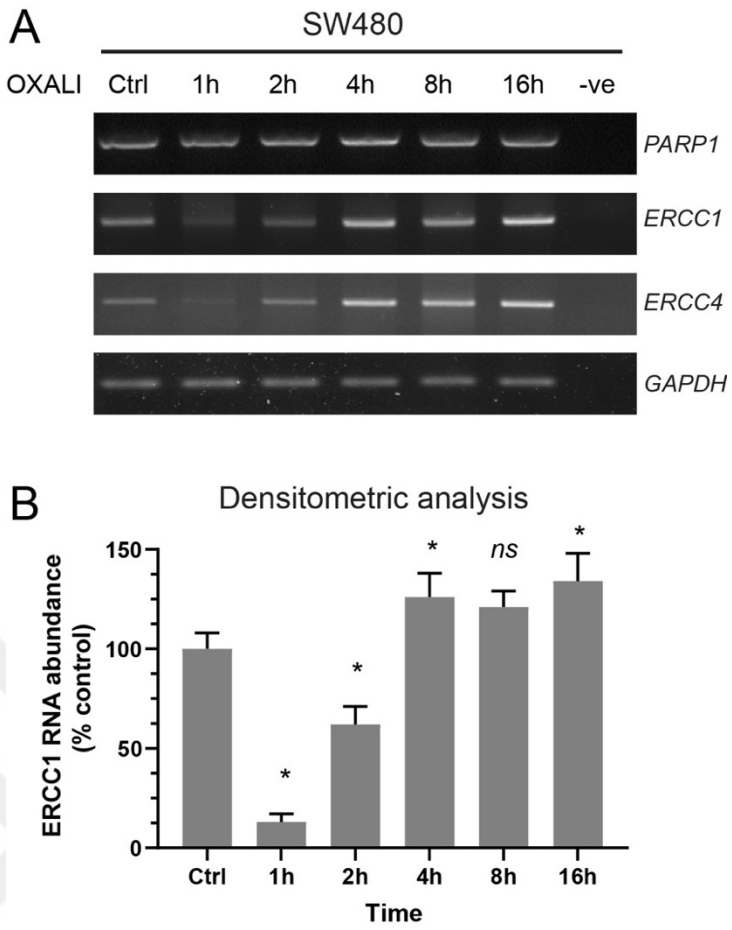

Figure 4.10: Kinetics response results of *ERCC1* gene expression upon oxaliplatin treatment. (A) Semi-quantitative RT-PCR analysis of *PARP1*, *ERCC1* and *ERCC4* genes. (B) Densitometric analysis was performed from 3 independent experiments for *ERCC1* gene using ImageJ and was presented as a graph.

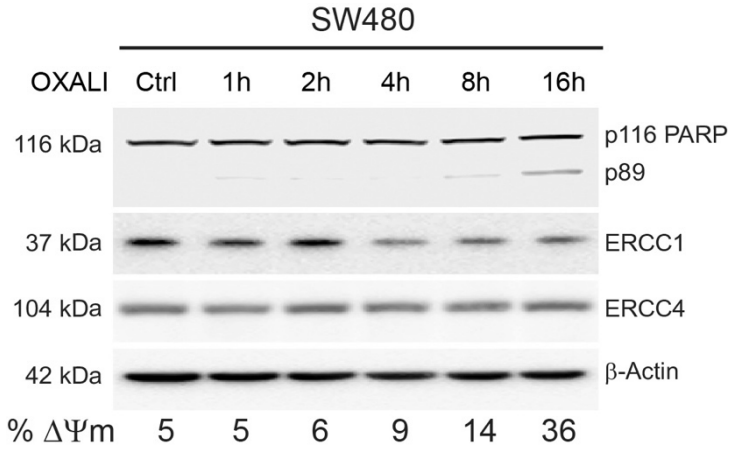

Figure 4.11: Kinetics response results of ERCC1 protein upon oxaliplatin treatment. Percentage of mitochondria depolarization was presented under each lane as ΔΨm.

4.6. ERCC1 Protein Degradation Analysis Using Proteosome Inhibitors

ERCC1 protein has been shown to undergo proteasomal degradation. Its deubiquitylase has been discovered as USP45 however its E3 ligase is still at large. It is also known from previous studies that ERCC1 is ubiquitylated however this modification does not target ERCC1 to proteasome. Importantly, all studies concerning ERCC1 protein stability or ubiquitylation were carried out in non-carcinoma cell lines [110, 111]. As more than 85% of cancers are carcinoma and our focus is on liver, breast, and colorectal cancers, we intended to examine the mechanism behind the decrease of ERCC1 level using the carcinoma cell line panel we already assessed in previous experiments. Our results confirmed that ERCC1 protein level increases with MG132 treatment in carcinoma cell lines consistent with the previous studies in non-carcinoma ones proving that it is targeted to proteasomal degradation (**Figure 4.12**).

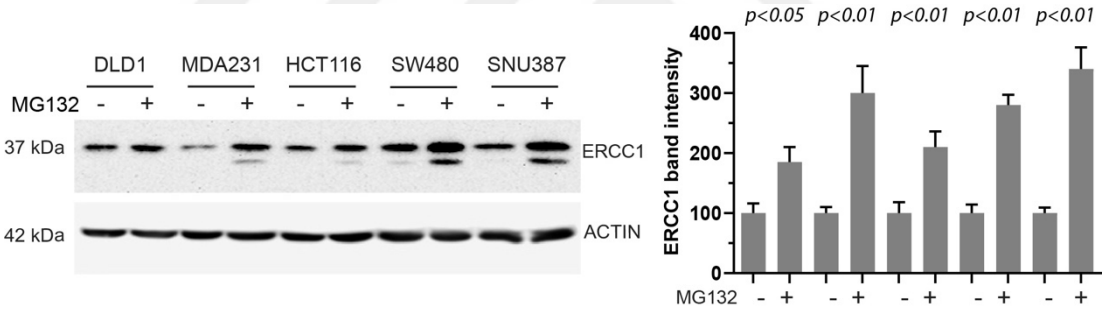

Figure 4.12: Western-blotting results of MG132-treated 5 carcinoma cell lines. Cells were incubated with 5uM MG132 for 6h. Quantification of ERCC1 bands was presented on the right.

4.6.1. Optimization of ERCC1 Stabilization through Proteosome Inhibition

ERCC1 overexpression causes cells to become resistant to apoptosis upon treatment with a variety of genotoxic agents. Our results suggest that ERCC1 protein goes down in the initial phases of apoptosis, before the "point of no return" in apoptosis is reached. We hypothesized that increasing endogenous levels of ERCC1 protein, for example by inhibiting proteosome, could delay/inhibit apoptosis, if ERCC1 is acting taking a part in DNA repair-apoptosis decision. For that reason, we selected to use the well

characterized proteosome inhibitor MG132. However, proteosome inhibition also induces apoptosis, especially during extended incubations such as we require during genotoxic drug treatments. Our criteria were to increase the level of ERCC1 significantly before adding DNA damaging agents using MG132 pre-treatment and to observe minimum PARP cleavage after DNA damage. We initially performed an optimization experiment where we treated SW480 at 2 different concentrations of MG132 at an early (4h) and late (20h) time points to observe the intrinsic apoptosis inducing activity of MG132. Our results showed that 0.5uM MG132 was sufficient to increase ERCC1 levels in SW480 cells at 4h, where we intend to add DNA damaging agents. This concentration of MG132 also did not induce significant apoptosis at either time (4h and 20h) point (**Figure 4.13**). Therefore, we selected this concentration for further experiments.

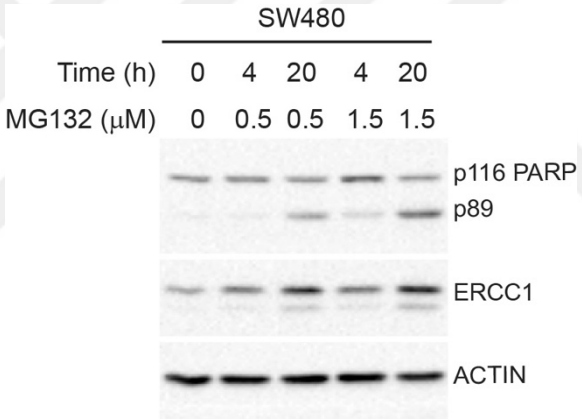

Figure 4.13: Optimization results of MG132 treatment for SW80 cell line. Cells were treated with 2 different concentrations of MG132 to determine optimized dose using for DNA damage induced apoptosis in controlled ERCC1 abundance.

4.6.2. Analysis of DNA Damage-Induced Apoptosis during Controlled ERCC1 Abundance

Once the right conditions were selected, we performed proteasome inhibition and evaluated the cellular response to DNA damaging agents by mitochondria depolarization and PARP cleavage. To that end, SW480 cells were pre-incubated MG132 (0.5μM) for 4h and then further incubated oxaliplatin (200μM) for 16h.

First and foremost, oxaliplatin decreased ERCC1 in line with our previous findings and MG132 stabilized ERCC1. We observed a minor protection from oxaliplatin induced apoptosis in SW480 cells upon MG132 treatment (**Figure 4.14**).

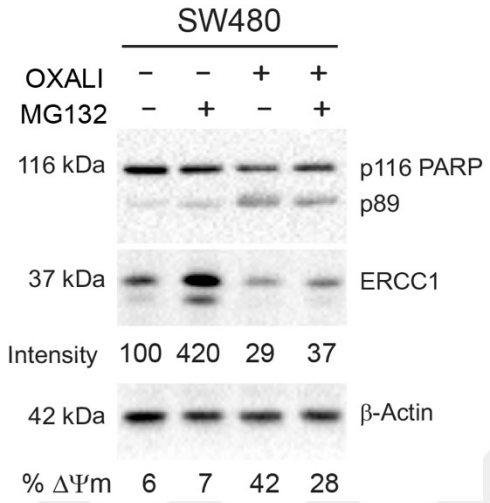

Figure 4.14: The effect of MG132 pre-treatment on oxaliplatin-treated SW480 cells.

4.6.3. Functional Contribution of Bortezomib Mediated ERCC1 Stabilization towards DNA Damage Response

Although haematological malignancies are treated with proteasome inhibitors such as Bortezomib, it never showed efficacy in solid cancers as part of a combination therapy where platinum derivatives are often the primary agent. We hypothesized that increase of ERCC1 abundance upon proteasome inhibition may be a reason for negative results obtained in clinical trials involving Bortezomib and platinum-based agents. To address this question, we first optimized the concentration of Bortezomib, in a similar manner we did for MG132. With the intention to identify the dose of Bortezomib that would stabilize the ERCC1 but not induce significant apoptosis after overnight incubation, we performed a concentration curve. To address this issue, SW480 cells were treated with 1, 3, 10, 30, 100nM Bortezomib and we observed that 1 and 3nM was not enough to increase ERCC1 levels. Although 10, 30 and 100nM stabilized ERCC1 protein, 100nM induced significant PARP cleavage. Thus, we identified the concentrations as 1, 10, 100nM as three main doses we used to carry out next experiment (**Figure 4.15**).

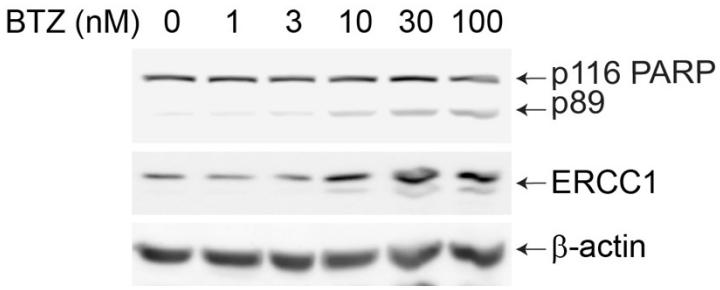

Figure 4.15: Optimization of Bortezomib dosage for SW480 cell line.

After optimization stage, we incubated SW480 cells with low (1nM), medium (10nM), and high (100nM) doses of Bortezomib in combination with oxaliplatin (200μM) and evaluated ERCC1 abundance and biochemical hallmarks of apoptosis. Our results showed that ERCC1 level increased in 10nM and 100nM Bortezomib treated samples only and there was no change for ERCC1 abundance in Bortezomib in 1nM-treated cells. We observed that low Bortezomib in combination with oxaliplatin had same amount PARP cleavage with oxaliplatin-only treated sample, so Bortezomib showed no additive effect, which was in line with unchanged ERCC1 abundance. Medium dose Bortezomib had an inhibitory effect on apoptosis compared to oxaliplatin-treated only cells (less PARP cleavage) and high dose Bortezomib showed more PARP cleavage than oxaliplatin-treated only sample (**Figure 4.16**). Therefore, proteasome inhibitors have an anti-synergistic action with oxaliplatin at conditions where they stabilize ERCC1 and do not induce apoptosis by themselves.

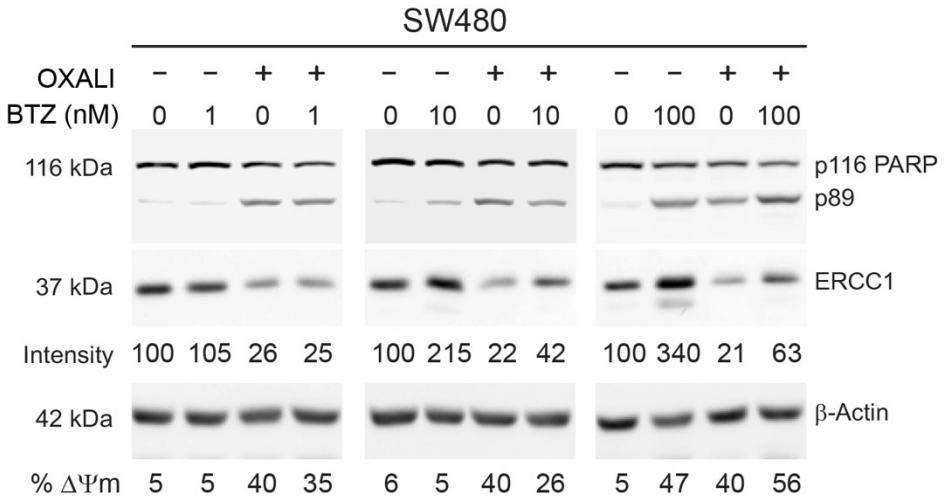

Figure 4.16: The effect of Bortezomib (BTZ) on oxaliplatin-treated SW480 cell line. Cells were pre-incubated for 6h with Bortezomib and then further incubated with oxaliplatin for 16h.

Another way of assessing combination (additive or synergistic) activity of 2 drugs is colony formation assay. As this is intended to assess the long-term outcomes, it mimics the activity of drugs (e.g., the viability of cells) in patients more effectively therefore more relevant to patient outcomes than the short term-apoptosis experiments. To find out the effects of Bortezomib on cell survival, we used the experimental setup such that SW480 cells were treated with oxaliplatin (1μM) and/or Bortezomib in different concentrations. Cells were counted and 2000 cells were seeded per dish and next day Bortezomib was added in different concentrations (1, 10 and 100nM). After 6h incubation, oxaliplatin was added for further 16h incubation. Media was changed next day and every 3-4 days. After 14 days, and when colonies were formed, cells were fixed, stained with crystal violet and images taken as shown in **Figure 4.17**. Colonies in each dish were counted. Control plates were set to 100% and the colony numbers in other plates were calculated as percentages compared to control.

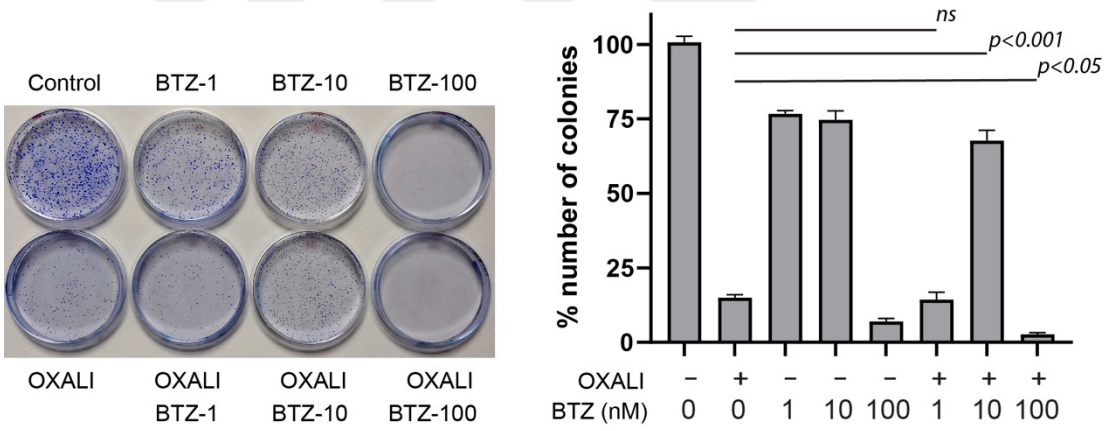

Figure 4.17: Colony formation assay results of oxaliplatin and/or Bortezomib-treated SW480 cell line. ns: nonsignificant.

Our results confirmed our earlier (apoptosis) assays where ERCC1 stabilization using proteasome inhibitors induced resistance to oxaliplatin induced apoptosis. This was apparent only at the concentration of proteasome inhibitor (10nM) that was not inducing apoptosis alone and but stabilizing ERCC1 protein significantly. At the lower concentration (1nM), addition of Bortezomib did not yield more apoptosis than oxaliplatin alone, neither ERCC1 was stabilized. At the highest concentration (100nM), Bortezomib alone induced apoptosis and its magnitude increased with oxaliplatin suggesting the overall effect produced is additive. Our results suggest, only

at the highest tolerable doses Bortezomib and oxaliplatin containing treatments will produce benefit to patients.

5. DISCUSSION AND CONCLUSIONS

NER is one of the earliest DNA maintenance-repair pathways, a process conserved from bacteria to high eukaryotes [25, 41]. However, despite functionally very conserved, the proteins involved in NER are very diverse in higher eukaryotes as compared to prokaryotes [25, 41]. NER component proteins were shown to play a role in eukaryotic events such as telomere maintenance and class switch recombination (CSR) suggesting they are evolved in fate decisions of diploid and chromosomally arranged cells [66, 67, 70, 71]. However, unlike eukaryote specific DNA repair pathways such as HR or NHEJ, there are no studies showing a direct function of a NER component protein in apoptosis. This is mainly due to technical problems associated with disconnecting DNA repair ability of a protein to its functional involvement in apoptosis. For example, when a cell receives excessive doses of UV, it will repair DNA damage faster if it has more ERCC1. From another point of view, the higher abundance of ERCC1 could be protecting the same cell from apoptosis by inhibiting pro-apoptotic proteins. Therefore, a simple knock-down or knock-out experiment will not be sufficiently informative. Detailed understanding of how NER is regulated during moderate (repairable) and excessive (unrepairable) DNA damage should give clues. Our current study is the first to investigate all components of NER proteins under a variety of apoptotic stimuli with a focus on timing and magnitude apoptosis. We intended to observe the changes in the abundance of NER component proteins using different DNA damaging agents and death receptor ligation, identifying when the changes are happening in relation to the "point of no return" of apoptosis signaling, which is mitochondria depolarization [112].

Our results suggest XPA, XPC and ERCC1 proteins were decreasing upon DNA damage induced apoptosis. The change in XPA is directly associated with the magnitude of apoptosis and the protein abundance can be restored with caspase inhibition. This suggests it is a target of effector caspases which is downstream of mitochondria depolarization. Therefore, XPA degradation is probably marking excessive-unrepairable DNA damage. NER pathway will be inhibited in the absence of XPA resulting in the use of all cellular energy in apoptosis, rather than DNA repair. The consequences of XPA decrease should be further analysed, especially in cancer

therapy. The decrease in XPC protein was only observed in doxorubicin treatments, independent of the magnitude of apoptosis. Further, caspase inhibition did not induce recovery of XPC protein abundance suggesting it is downregulated specifically upon topoisomerase inhibition conditions, independent of apoptosis, as a result of stalled transcription or DNA replication and single or double strand DNA breaks. As XPC is the key NER component, detecting DNA helix distortions in the early phases of NER, its decrease upon doxorubicin treatment could be as a result of diverting cellular energy from NER to NHEJ or HR because the damage induced by doxorubicin is mostly repaired by these pathways [23]. Additionally, the inhibition of topoisomerase by doxorubicin could be inducing torsional stress on DNA, especially around replication forks or actively transcribed DNA segments, which could trigger TC-NER in the absence of NER specific DNA adducts (DNA crosslinks). In TC-NER, stalled RNA polymerase acts as a signal for NER assembly, and it proceeds without the need of XPC or XPE. It is highly probable that NHEJ or HR have a link to inhibit GG-NER activity in conditions where XPC is not required such as TC-NER, or at conditions where the damage exists but not in the merit of NER (e.g., single or double strand breaks). This can be achieved by selective XPC degradation. The detailed mechanism of XPC degradation upon topoisomerase inhibition requires validation with multiple small molecules, using a variety of genetically defined (e.g., p53 mutant, wild type, etc.) and genetically modified (e.g., inducible knock down) cell lines.

In our assays, the most striking and apoptosis linked change observed in a NER component protein was a decrease in ERCC1 abundance. The observation was consistent among a variety of cancer cell lines and with different DNA damaging agents but not detected during death receptor mediated apoptosis. Most importantly, DNA damage induced ERCC1 decrease was happening at the protein level and before the point of no return in apoptosis has been achieved suggesting it is a deciding effect rather than a bystander of apoptosis. Our experimental approach will, allow using time points and concentrations of drugs, that one can precisely map the function of ERCC1 in apoptosis or DNA repair. For example, if the intention is to study the role of ERCC1 in DNA repair, concentrations of drugs used should not be inducing ERCC1 decrease at a time before mitochondria is depolarized. Therefore, ERCC1 decrease can be used as an indicator of cellular decision of “to repair” or “to die”. Our study also hints the mechanism of ERCC1 decrease as proteasomal degradation. Ubiquitylation is the main

mechanism of targeted proteasomal degradation [113]. ERCC1 is known to be ubiquitylated however, this modification does not seem to control its stability but its function [110, 111]. Recent studies suggest the presence of structure associated proteasomal degradation pathways as shown for Fos family of transcription factors [114]. An important follow up study should be identifying the precise mechanism of ERCC1 downregulation upon excessive DNA damage, which will be one of my next challenges. As ERCC1 is also known to play a role in telomere maintenance and CSR, its control can be important in ageing and adaptive immunity. The most important use of selective ERCC1 degradation will be during chemotherapy of cancer. Cisplatin, oxaliplatin and mitomycin c are commonly used chemotherapeutic agents, all of which induce DNA damage that is repaired by NER [115]. Overexpression of NER component genes, especially ERCC1, have been assessed and shown to correlate with chemoresistance in colorectal, lung, bladder, ovarian and testicular cancers [90-93, 107]. A controlled and cancer specific decrease in ERCC1 protein will certainly improve chemotherapy response in the cancers listed above. The understanding of DNA repair will create benefit in cancer treatment, ageing and immune disorders.

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BIOGRAPHY

Şule ERDEMİR SAYAN holds a BSc. Degree in Biology from Istanbul University and a MSc. Degree in Molecular Biology-Genetics and Biotechnology from Istanbul Technical University, awarded in 2009 and 2013, respectively. She is currently pursuing a Ph.D. Degree in Molecular Biology and Genetics at Gebze Technical University.

PUBLICATIONS AND PRESENTATIONS FROM THE THESIS

Erdemir Sayan, S., Sreekumar, R., Bhome, R., Mirnezami, A., Yagci, T., Sayan, A. E., (2024), "ERCC1 abundance is an indicator of DNA repair-apoptosis decision upon DNA damage", Cell Death Discovery, 10(1), 47.

Erdemir Sayan, S., Yagci, T., Sayan, A. E., (2022), "ERCC1 abundance controls apoptosis-DNA repair decision upon DNA damage", University of Southampton Cancer Sciences Conference, Southampton, UK, 23 November.

APPENDICES

APPENDIX-A: DNA AND PROTEIN MARKERS

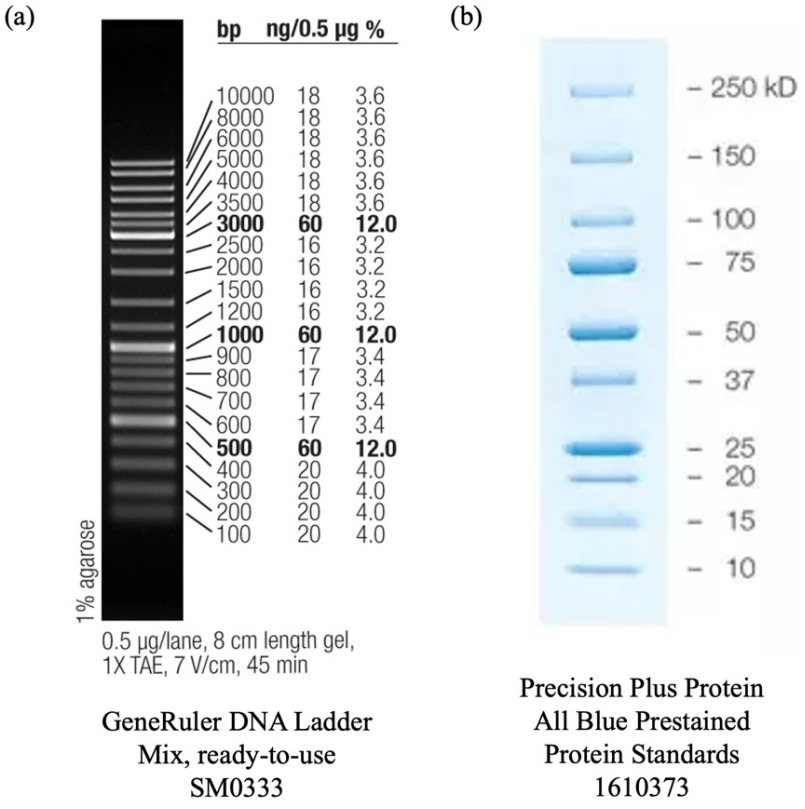

Figure A.1: DNA and protein markers used during the study. (a) DNA marker, (b) Protein marker. DNA band sizes are shown in bp (base pair). Protein band sizes are shown in kD (kilodalton).

APPENDIX-B: PERMISSIONS FOR COPYRIGHTED MATERIALS

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