

**Characterization of the Functional Roles of the E3
Ligase HUWE1 in the Regulation of Poly [ADP-ribose]
Polymerase 1 (PARP-1) under DNA Damage**

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Abstract

The Poly (ADP-ribose) polymerase 1 (PARP-1) is responsible for synthesis of polymeric ADP-ribose chain on substrate proteins associated with DNA damage repair. PARP-1 overexpression has been associated with rapid proliferation and metastasis in cancers. Therefore, investigating how PARP-1 is finely regulated under DNA stress conditions is of important to better understand the physiological and pathological role of PARP-1. Ubiquitylation is a protein post-translational modification and the key bioprocess for the regulation of PARP-1 functionally. Recently, ubiquitin E3 ligase HUWE1 (HECT, UBA and WWE domain containing 1) was reported to be involved in DNA damage response. HUWE1 also possesses a functional WWE domain, which is a structural motif to recognize the ADP-ribose unit of PAR polymers. In this study, the functional roles of HUWE1 in the regulation of PARP-1 were characterized. This study reveals (1) HUWE1 could bind to cellular PAR polymers through its WWE domain. (2) As PARP-1 is modified through auto-PARylation in the cell, HUWE1 could interact with the PARylated PARP-1 via WWE domain. (3) HUWE1 could regulate PARP1 stability through ubiquitination. This study provides mechanistic insight on the regulation and the function of these two proteins in both biological and pathological conditions.

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Table of Contents

Abstract.....	ii
Acknowledgement	iii
Table of Contents.....	iv
List of Figures	vi
List of Tables	vii
List of abbreviations	viii
Chapter 1 : Introduction	1
1.1 General review of major types of DNA damage	2
1.2 DNA damage induced by chemotherapy compounds.....	4
1.3 The role of PARP-1 in DNA damage	5
1.4 The consequence of PARP-1 overactivation and its implication in pathogenesis and therapy	11
1.5 Regulation of PARP-1 by ubiquitin-proteasome system.....	15
1.6 The role of HUWE1 in DNA damage	18
1.7 Research Rationale	20
1.8 research aims.....	22
Chapter 2 : Material and methods	25
2.1 Cell Culture.....	26
2.2 Plasmid transformation and amplification.....	26
2.3 Plasmid transfection to overexpress HUWE1 and WWE mutants	26
2.4 Cell treatment (DNA damage induction, PARP-1 inhibition, and MG 132 treatment).....	27
2.5 Co-immunoprecipitation	27
2.6 Fractionation preparation.....	28
2.7 ShRNA knockdown HUWE1	29
2.8 Detection of HUWE1 and PARP1 mRNA expression using qPCR.....	31
2.8.1 RNA isolation.....	31
2.8.2 cDNA preparation	32
2.8.3 qPCR reaction.....	32
2.9 Immunoblotting	33
2.10 Immunofluorescence (IF) assay.....	34
2.11 Cell titer Glo 2.0 cytotoxicity assay.....	35
2.12 Statistics	35
Chapter 3 : Results	37

3.1 HUWE1 could directly interact with PAR	38
3.2 HUWE1 can interact with PARP-1 via WWE domain.....	41
3.3 HUWE1 is a E3 ligase for PARP-1 and mediates PARP-1 Ubiquitination.....	43
3.4 HUWE1 knockdown increased the PARP-1 protein level in U2OS cells.....	45
3.5 HUWE1 knockdown increased the nuclear PARP-1 level in U2OS cells.	48
3.6 HUWE1 knockdown resulted in increased BRCA1 and decreased 53BP1 foci in the nuclei following DNA damage.....	51
3.7 HUWE1 and PARP-1 showed a reversed protein expression pattern following induction of DNA damage by etoposide.....	55
3.8 The effect of HUWE1 KD on DNA damage induced cell death in the U2OS cell	58
Chapter 4 : Discussion and future directions	60
4.1 HUWE1 is an E3 ligase for PARP-1	62
4.2 HUWE1 KD can increase PARP-1 protein stability	64
4.3 HUWE1 was involved in both HR and NHEJ repair pathway	64
4.4 HUWE1 negatively regulated PARP-1 level during DNA lesion	65
References	68
Appendix	76
Supplementary data.....	77

List of Figures

Figure 1.1 The PARP-1 participates in DNA damage repair pathways.....	8
Figure 1.2 The schematic view of PARylation and PARP-1 in DNA damage.....	11
Figure 1.3 The protein signaling pathway involved in parthanatos.....	13
Figure 1.4 The ubiquitination-proteasome degradation pathway.	17
Figure 1.5 Schematic diagram of the structural domains of HUWE1.	20
Figure 2.1 pGIPZ shRNA vector expression system used for shHUWE1-G10 and shHUWE1-F6 stably knocked down cell lines	30
Figure 3.1 HUWE1 interacted with PAR via HUWE1 WWE domain.....	40
Figure 3.2 HUWE1 interacted with PARP-1 via HUWE1 WWE domain	42
Figure 3.3 HUWE1 is a E3 ligase for PARP-1 and mediated PARP-1 ubiquitination.....	44
Figure 3.4 HUWE1 knockdown stabilized PARP-1 in protein level.....	47
Figure 3.5 HUWE1 knockdown stabilized PARP-1 protein.....	50
Figure 3.6 HUWE1 knockdown resulted increased BRCA1 and decreased 53BP1 protein stabilities.....	54
Figure 3.7 HUWE1 and PARP-1 expression levels in response to DDR	57
Figure 4.1 schematic summary of the research.....	62

List of Tables

Table 1.1 Representative DNA damages and the associated cellular repair pathways.....	4
Table 2.1 The reaction mix for cDNA synthesis.....	32
Table 2.2 Primers used in the RT-qPCR reaction	33
Table 2.3 List of antibodies and associated dilution preparation.....	36

List of abbreviations

AIF, Apoptosis inducing factor

BER, Base excision repair

BRCA, Breast cancer gene

DDR, DNA damage response

DPT, Deoxypodophyllotoxin

DSB, Double strand breakage

HR, Homologous recombination repair

IF, Immunofluorescence microscopy

IR, Ionizing radiation

MMR, DNA mismatch repair

NER, Nucleotide excision repair

NHEJ, Nonhomologous end joining repair

PAR, Poly (ADP-ribose)

PARP-1, Poly (ADP-ribose) polymerase 1

PCR, Polymerase chain reaction

ROS, Reactive oxygen species

ShRNA, Small hairpin RNA

Top II, Topoisomerase II

Ub, Ubiquitin

UV, Ultraviolet radiation

VE, Empty vector

WB, Western blot

Chapter 1 : Introduction

1.1 General review of major types of DNA damage

Deoxynucleotide acid (DNA), apart from several RNA viruses, carries genetic information and exists in all living organisms. DNA has a double helix structure comprised of four types of nucleotides, cytosine (C), guanine (G), adenine (A) and thymine (T), connected by a sugar-phosphate backbone (Barchiesi et al., 2021; Deweese et al., 2008; Travers and Muskhelishvili, 2015). Cellular DNA can be exposed to a variety of damaging agents, from both endogenous and exogenous sources, leading to different types of DNA lesions. Such DNA damage affects normal function of a cell, if left unrepaired, could result in senescence or cell death.

The exogenous DNA damage include ultraviolet (UV) radiation, alkylating agents, aromatic amine agitation, and ionizing radiation (IR) (Devatkal et al., 2013; Gopal and Guruprasad, 2010; Lecoite et al., 2007; Sheehy et al., 2020a; Yoon et al., 2021). The ultraviolet radiation (UV-A 320-400nm, UV-B 290-320nm and UV-C 190-290nm) damages DNA primarily by causing covalent linkage between adjacent pyrimidines. The most common ones are cyclobutene pyrimidine dimers and pyrimidine (6-4) pyrimidone photoproducts (Gopal and Guruprasad, 2010).

The alkylating agents include chemicals such as methyl-methanesulfonate and methyl-nitrosourea (Travers and Muskhelishvili, 2015). These reagents can cause DNA damage by methylating the N7 of deoxyguanine and N3 of deoxyadenine, resulting in arrest of DNA replication forks (Lecoite et al., 2007; Travers and Muskhelishvili, 2015). Aromatic amines are organic compounds with at least one aromatic ring attached to an amine. The most well-known aromatic amines are N-2-acetyl-aminoflourene (AAF) and N-2-aminoflourene (AF), which can be esterized and subsequently cause DNA lesions by disrupting the DNA structure and inserting

into the DNA helical structure to replace the C8 oxyguanine, which will eventually lead to base substitution and frameshift mutation (Devatkal et al., 2013; Deweese et al., 2008; Dey et al., 2012). High dose of IR has two effects, (1) to cause a spectrum of DNA base lesions and subsequently induce both DNA single and double-strand breakage, and (2) to induce the production of the reactive oxygen species (ROS) (Hong et al., 2020; Mateo Gonzalez-Roman et al., 2021; Roh and So, 2017). The ROS can indirectly cause DNA damage by generating apurinic and apyrimidinic sites in the DNA (Mateo Gonzalez-Roman et al., 2021).

ROS is critical for maintaining intercellular signaling when it is under fine control and low level. However, excessive amount of ROS can cause serial oxidative base lesions. The ROS can attack both the DNA bases and the DNA backbone (Mateo Gonzalez-Roman et al., 2021). A great number of diseases such as Alzheimer's disease, Parkinson's disease, diabetes, cardiovascular diseases, and certain types of cancers are involved in ROS overactivity. (Raavi et al., 2021)

The endogenous DNA damage can be induced by different intracellular processes, including replication errors, spontaneous base deamination, oxidative DNA damage, DNA methylation and topoisomerase enzymes (Top I and Top II) dysfunction (Dey et al., 2012; Sheehy et al., 2020b). Replication errors could be resulted from unexpected nucleotide base insertions or deletions in the DNA strand synthesis. The spontaneous base deamination refers to the type of DNA damage in which 5-methyl cytosine, cytosine, guanine, and adenine lose their exocyclic amines to form thymidine, uracil, xanthine, and hypoxanthine respectively. It is one of the major reasons for GC to TA mutation (Dey et al., 2012; Sheehy et al., 2020b).

Table 1.1 Representative DNA damages and the associated cellular repair pathways.

DDR agents	Environmental toxins (Alkylating and aromatic agents)	ROS associated damages	Ionizing radiation	UV radiation
DNA lesion types	Mismatch mutation Uracil mutation Abasic site mutation Nucleotide mutation	Single strand breakage Double strand breakage	Predominantly Double Strand Breakage Also Cause Single Strand Breakage	Cyclobutane pyrimidine dimers 6-4 photoproducts
DNA repair pathways	Mismatch repair Base excision repair	Base excision repair Single and double strand breakage repair	Base excision repair Translesion synthesis DSB repair	Nucleotide excision repair Base excision repair

1.2 DNA damage induced by chemotherapy compounds

Cancer cells are characterized by rapid proliferation and cell division which require repeated runs of DNA replication. One category of chemotherapeutic drugs directly target and compromise DNA replication, which eventually cause cell death. This includes the chemotherapy compounds that target topoisomerase enzymes (Jackson and Bartek, 2009; Lin et al., 2018; Luepker, 2011; Pai et al., 2017). Topoisomerase is a class of essential enzymes involved in resolving DNA supercoils to maintain normal DNA topology. The major role of topoisomerase enzymes is to maintain the topological DNA structure during the replication or transcription processes (Jeggo and Lobrich, 2007; Leung et al., 2019). During DNA replication, the separation of two strands will generate positively supercoiled DNA ahead of the replication fork. Accumulating torsional strain resulting from overwound DNA could block DNA replication and cause DNA breakage. Therefore, topoisomerase is essential for the normal function of the cell (Chandran et al., 2015; Edmond et al., 2017).

There are two major types of topoisomerases. Type I topoisomerase, or Top I, cuts the phosphodiester DNA backbones in only one strand of the DNA double helix in an ATP

independent manner, whereas Type II topoisomerase, or Top II, cleaves both two DNA strands in ATP dependent manner (Edmond et al., 2017; Mukherjee Chatterjee et al., 2018). After the completion of replication or transcription, the topoisomerase must be removed from DNA so that the DNA can re-ligase. Inhibition of topoisomerase leads to single- and double- strand DNA breaks during the replication or transcription processes, which sensitize cells undergoing apoptosis (Glass et al., 2006; Zhang et al., 2019). Therefore, a class of anti-cancer drugs have been developed to specifically target topoisomerase to induce apoptosis in cancer cells, as rapid proliferation cancer cells are more sensitive to the inhibition of topoisomerase.

Etoposide, the chemical formula $C_{29}H_{32}O_{13}$, is one of the earliest selective topoisomerase II (Top II) inhibitors, approved by FDA for over three decades (Glass et al., 2006). Its main function is to stabilize the Top II-DNA complex by covalent binding of Top II to the unwounded DNA. During DNA replication, Top II is recruited to the replicated DNA sites to unwind DNA supercoil structure (Glass et al., 2006). Etoposide prevents Top II from being removed from DNA to inhibit the unwounded DNA re-ligation with the complimentary DNA strands (Glass et al., 2006; Leung et al., 2019). The consequence of this is to induce DNA double strand breakage (DSB) in the Etoposide treated cells (Chandran et al., 2015). Low doses of Etoposide induces DSB in DNA replication, whereas high doses of etoposide induces DSB in both replication and transcription (Jeggo and Lobrich, 2007). In this study, Etoposide is used to induce DSB for characterization of the signaling pathways triggered by the DNA damage.

1.3 The role of PARP-1 in DNA damage

Cells are constantly exposed to various stresses which are prone to cause DNA damages. Accumulation of DNA lesions can harm normal cellular functions by mutating DNA sequences,

affecting DNA stability and causing DNA breakage (Caglayan and Wilson, 2015). If these DNA errors are processed incorrectly, the lesions will lead to mutations or wide-scale genomic aberrations, which will eventually threaten the viability of the cell (Jackson and Bartek, 2009). Therefore, precise DNA damage repair in cells is critical to the viability of the living organism.

There are several mechanisms that the cell uses to sense and repair DNA damages, which is generally called DNA damage repair response (DDR). Base excision repair (BER), nucleotide excision repair (NER), DNA mismatch repair (MMR), homologous recombination repair (HR) and nonhomologous end joining repair (NHEJ), are five major types of DNA damage repair (Bartoli-Leonard et al., 2021; Cui et al., 2021; He et al., 2015; Khafaei et al., 2015; Wu and Wang, 2021; Zhao et al., 2021). BER repairs lesions occurring in small, non-helical twisted DNA bases, which requires DNA glycosylase, AP endonuclease, exonuclease and NA polymerase I to remove the base lesion, substitute with the correct base, and complete the repair (Bartoli-Leonard et al., 2021; Khafaei et al., 2015). MMR refers to a mechanism which is highly strand specific to remove the errors from newly synthesized cells (Khafaei et al., 2015). During DNA replication or recombination, errors can occur such as unexpected base insertion, deletion, or mis-incorporation. To prevent the errors from being passed into daughter cells, MMR will be initiated to correct these errors (He et al., 2015). NER is the most important DNA repair mechanism to repair UV induced DNA lesion. The UV radiation can induce bulky DNA lesions such as thymine dimers and 6,4-photoproducts. A small fragment of DNA will be removed, and the DNA polymerase will use the undamaged single DNA strand as the template to fix the errors (Erkizan et al., 2021). HR is the mechanism which functions to repair DSB. When both strand of DNA are damaged, the cells developed a mechanism that using the DNA from homologous chromatin as a template to fix the errors (He et al., 2015). NHEJ, similarly to HR, is also activated in DNA DSB.

However, the NHEJ repairs DNA by microhomologies instead of using homologous DNA strand. Due to this property, the errors happened in NHEJ can cause translocation or telomere fusion which are the biomarkers for cancer development (Han et al., 2021; He et al., 2015; Khafaei et al., 2015; Wu and Wang, 2021).

Regardless of the type of DNA damage repair pathway, following DNA damage, many DNA lesion associated proteins will be recruited by the cell onto the damage sites. Poly (ADP-Ribose) Polymerase 1 (PARP-1) is one of the early DDR proteins recruited onto the lesion site (Caglayan and Wilson, 2015; Cui et al., 2021; Erkizan et al., 2021; Rodriguez et al., 2021; Seo et al., 2021). It has been clear that both DNA damaging agents, such as etoposide, and IR can cause increased expression of the PARP-1 protein (Rodriguez et al., 2021; Seo et al., 2021), (Figure 1.1). The PARP-1 can further regulate and activate other DDR associated proteins such as p-ATM, p53 and γ H2AX (Di Emidio et al., 2021; Han et al., 2021; He et al., 2015; Seo et al., 2021; Su et al., 2021; Zhao et al., 2021).

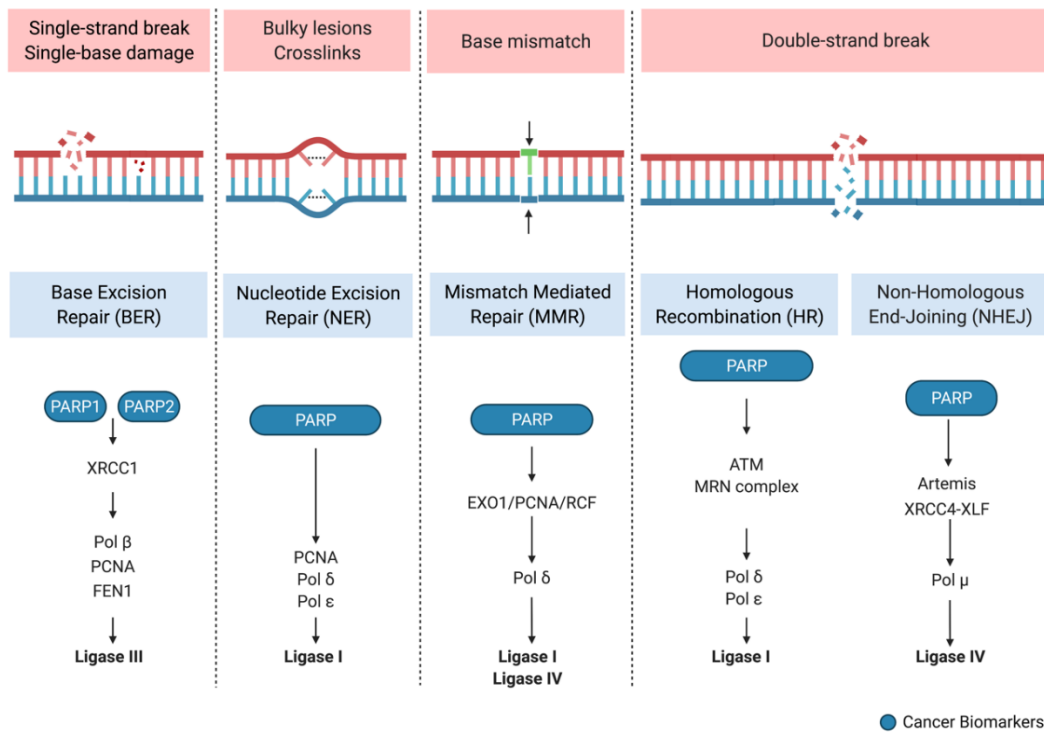


Figure 1.1 The PARP-1 participates in DNA damage repair pathways. PARP-1 is one of the proteins recruited to DNA lesions and becomes activated by binding to DNA. PARP-1 is involved in five major DNA damage responses include BER, NER, MMR, HR and NHEJ. (Di Emidio et al., 2021; Han et al., 2021; He et al., 2015; Houl et al., 2019; Khafaei et al., 2015; Niazi et al., 2021; Seo et al., 2021; Su et al., 2021; Zhao et al., 2021).

PARP-1 belongs to the Poly (ADP-Ribose) Polymerase family proteins, which is comprised of 18 members. They are generally divided into two groups, the PARPs (PARP 3, 4, 6, 7, 8, 10, 11, 12, 14 and 15) for mono ADP-ribosylation (MARylation) and the PARPs (PARP-1, 2, 5) for PARylation (Cui et al., 2021; Di Emidio et al., 2021).

PARP-1, also known as NAD⁺ dependent ADP-ribosyltransferase 1, is the first protein that was identified within PARP family. It is a 116 kDa protein, encoded by the gene PARP-1 located on chromosome 1q42.12. The PARP-1 protein contains three main domains. The first is a DNA binding domain that contains three Zinc fingers. As the name indicated, this domain is responsible for sensing and binding of PARP-1 to DNA lesions. The second is a PARP-1 auto-

modification region, including the BRCA1 C-terminal (BRCT) domain and the tryptophan-glycine-arginine-rich (WGR) domain, found to be associated with PARP-1 auto-PARylation. The last is the catalytic domain that functions to catalyze the synthesis of polymeric ADP-ribose chains (PAR chains) from NAD⁺. Over 90% of nuclear PAR chains are synthesized by PARP-1, the rests are synthesized by protein members from PARP family such as PARP-2. The major difference between PARP-1 and PARP-2 is that the PARP-2 lacks an N-terminal DNA binding domain. However, PARP-2 exhibits a compensation ability for PAR synthesis when PARP-1 is depleted. PARP-5 is predominantly involved in WNT and beta-catenin signaling pathway by PARylating the AXIN1 and AXIN2 proteins (Cui et al., 2021; Hadisaputri et al., 2021; He et al., 2015; Niazi et al., 2021).

MARylation, which attaches mono ADP-ribose to the target protein, is involved in many critical bioactivities such as RNA modification, cytoskeleton protein (microtubules) stabilities, and immune responses (Di Emidio et al., 2021; Erkizan et al., 2021; Niazi et al., 2021; Wu and Wang, 2021). However, the MARylation does not show a significant and direct impact on DDR. Compared to MARylation, PARylation, referring to covalent linkage of highly negatively charged PAR chains to the substrate, has a close association with DDR initiated bioactivities (Figure 1.2). For example, in DNA single-strand breakage (SSB), PAR chains can bind to the BRCA1-C-terminal (BRCT) domain of the X-ray repair cross complementing protein (XRCC1) to initiate early recruitment response to DNA lesions. Also, the PARP-1 has been shown to interact with many fundamental regulatory SSB proteins such as 8-oxoguanine glycosylase 1 (OGG1), XRCC1, DNA polymerase beta (DNAP), DNA ligase III and proliferating cell nuclear antigen (PCNA). In DSB repair, such as NHEJ, PARP-1 can interact with polynucleotide kinase/phosphatase (PNKP) which is a key regulator for NHEJ. Though PARP-1 is not required

to initiate HR, it can bind to RAD51 and MRE11 which are two major proteins in HR initiation and HR response (Gero et al., 2014; Glass et al., 2006; Hopp et al., 2019; Pai et al., 2017).

PARylation is a key bioprocess that regulates DDR through modifying many important DNA damage repair proteins including OGG1, XRCC1, DNAP, DNA ligase III, PCNA and BNKP (Gu et al., 2020; Jackson and Bartek, 2009; Kotalczyk et al., 2020; Luepker, 2011; Mukherjee Chatterjee et al., 2018; Pai et al., 2017; Zhang et al., 2019). The defective PARylation can affect the normal recruitment and function of these proteins in DNA damage response and repair, and result in detrimental consequences to the cell, such as decreased level of genomic stability, chromatin dysregulation (aneuploidy), centrosome amplification and telomere shortening (Hadisaputri et al., 2021; Hopp et al., 2019; Houl et al., 2019). In extreme conditions, such as with PARP-1 and PARP-2 double knockout, abrogation of PARylation can cause embryonic lethality (Hadisaputri et al., 2021; Hopp et al., 2019; Houl et al., 2019).

ADP-ribosylation is a reversible post-translational modification. While PARP family proteins covalently attach mono or poly ADP-ribose to target proteins, the modifications can be removed by Poly-ADP ribose glycohydrolase (PARG) and ADP ribose glycohydrolase 3 (ARH3) (Hopp et al., 2019; Niazi et al., 2021; Zhao et al., 2021). Though both PARG and ARH3 reverse ADP-ribosylation, they exhibit clear differences. The PARG is primarily responsible for the degradation of PAR O-glycosidic bonds within PARylation, and it has very little effect on MARylation. In contrast, ARH3 showed low activity for the removal of PAR from PARylation compared to PARG, but it can efficiently remove serine linked MAR (Abplanalp et al., 2017; Leung et al., 2019; Zhao et al., 2021).

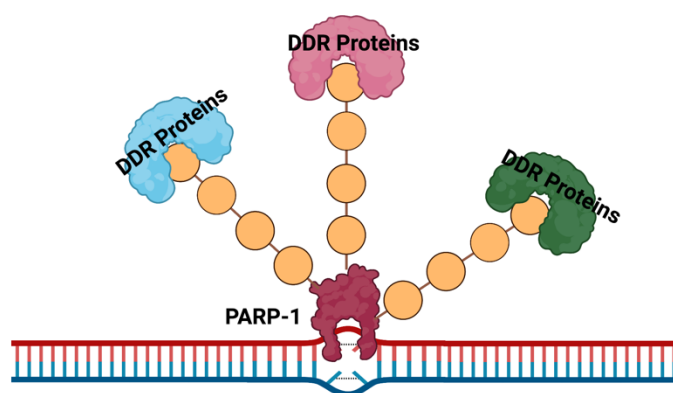
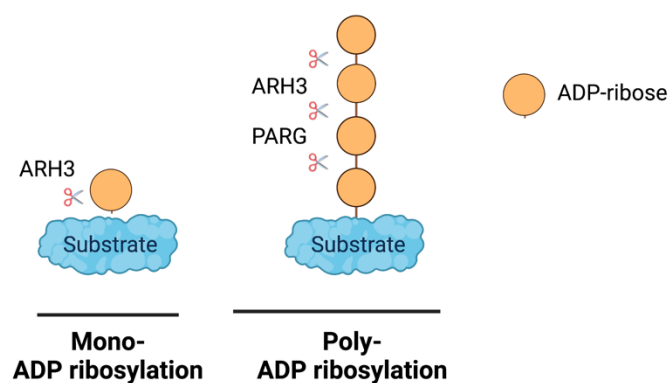


Figure 1.2 The schematic view of PARylation and PARP-1 in DNA damage. The structural diagram of MARYlation, Parylation and the associated protein which are recruited by PARP-1 and other DNA damage associated proteins (Abplanalp et al., 2017; Cui et al., 2021; Di Emidio et al., 2021; Hadisaputri et al., 2021).

1.4 The consequence of PARP-1 overactivation and its implication in pathogenesis and therapy

The most direct role of PARP-1 activation is to synthesis PAR for PARylation. In this process, the NAD^+ was used as the substrate to synthesis PAR with the consumption of ATP. PARP-1 is generally considered to function as a protective protein in DNA damage repair for maintaining cellular homeostasis (Cui et al., 2021; Hadisaputri et al., 2021). However, recent

studies also suggested that the overactivation of PARP-1 could be detrimental to the cells in an irreversible manner called parthanatos (Koehler et al., 2021; Lv et al., 2021; Xue et al., 2021).

In parthanatos, the overactivation of PARP-1 leads to massive PARylation. The PAR chains, by an unknown mechanism, penetrate the nuclear membrane and are translocated into the cytosol. In the cytoplasm, the PAR could interact with mitochondrial apoptosis inducing factor (AIF), and the binding between PAR and AIF is ATP and NAD⁺ independent (Koehler et al., 2021; Lv et al., 2021) (Figure 1.3). One experiment suggested that the binding is not compromised when supplying the cells with additional NAD⁺ or ATP. The interaction between PAR and AIF triggers the mitochondria depolarization, and the PAR–AIF complex could then migrate back into nucleus (Xue et al., 2021). Once the complex is in the nucleus, the AIF is released and bind to DNA to cause irreversible DNA fragmentation. More interestingly, this process does not require the activation of caspase 3 which is the biomarker for apoptosis (Xue et al., 2021).

Parthanatos

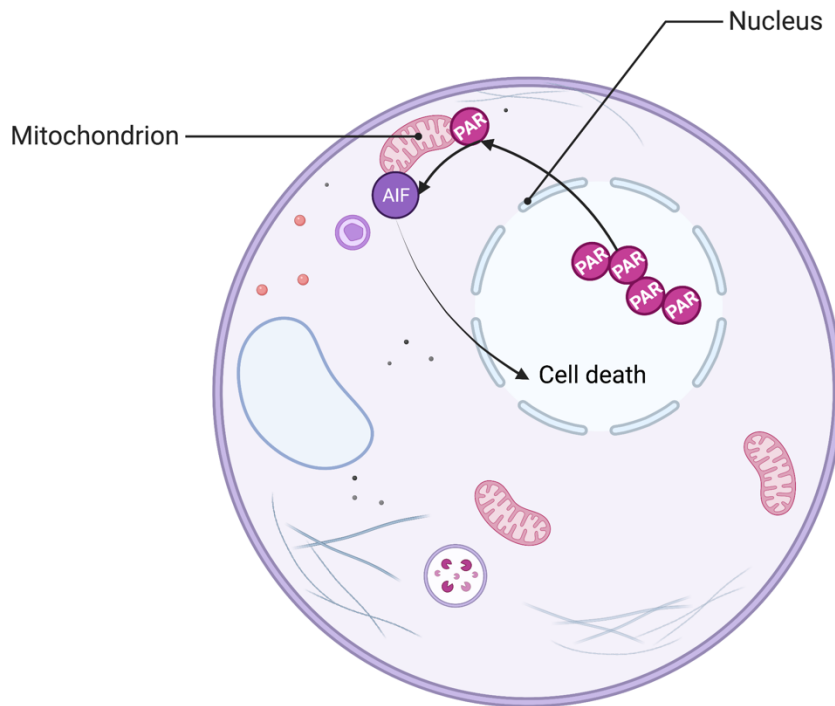


Figure 1.3 The protein signaling pathway involved in parthanatos. Overactivation of PARP-1 will lead to the overproduction of PAR. The PAR can migrate from nucleus into cytosol from where it binds to mitochondria. The mitochondria will subsequently release Apoptosis Inducing Factor (AIF) and the AIF-PAR complex will migrate back into nucleus. After entering the nucleus, AIF will cause DNA fragmentation and lead to irreversible cell death. The entire process is independent of caspase family proteins mediated apoptosis (Cao et al., 2019; Koehler et al., 2021; Xue et al., 2021).

Ma et al. reported that in U87 and SF126 glioma cells treated with Deoxypodophyllotoxin (DPT), an herbal extraction which can bind to tubulin directly to arrest cells in G2/M phase, the PARP-1 level increased drastically (Ma et al., 2016). This led to overproduction of PAR and translocation of the AIF from mitochondria to nucleus to cause irreversible cell death. Indeed, when the researchers applied PARP-1 inhibitor 3-Aminobenzamide prior to DPT treatment, the glioma cells were less susceptible to DPT induced cell death (Ma et al., 2016). This experiment

implies that the PARP-1 activation could be a protective mechanism to mitigate the impact of DNA damage, however, the overactivation of PARP-1 is detrimental to the cells by sensitizing cells for apoptosis.

PARP-1 KO animal models also suggested a binary role of PARP-1 activity in pathological conditions (Cao et al., 2019). The PARP-1 KO can sensitize tumor cells undergoing apoptosis. As the rapid proliferating tumor cells require a high level of bioactivity of PARP-1 to repair lesions, accumulation of DNA lesions resulting from impaired DNA repair in the PARP-1 KO cells could trigger cellular apoptosis. However, PARP-1 KO can protect mice from diseases which are often associated with PARP-1 overactivation such as lipopolysaccharide induced shock, ischemic injury, stroke and excitotoxicity (King et al., 2021; Ma et al., 2016).

Due to its association with tumorigenesis, many drugs have been developed to inhibit PARP-1 activity either as a single agent or in combination with other chemotherapeutic drugs. PARP-1 inhibitor as a cancer therapeutic agent was recently approved clinically to treat BRCA1 or BRCA2 mutated breast cancers and ovarian cancers (Cao et al., 2019; King et al., 2021). The PARP-1 inhibitors showed over 100-fold sensitivity in BRCA1/2 deficiency cells compared to no BRCA1/2 mutation cancer cells as a consequence of synthetic lethality effect (Cao et al., 2019; King et al., 2021). The synthetic lethality refers to the deadly effect to the cells when two or more genes have been mutated at the same time, and the single gene mutation causes minimal effect alone. As there are two mechanisms for cancer cells to deal with DSB, HR and NHEJ, the PARP-1 is believed to be a pivotal protein involved in NHEJ and BRCA1/2 are also the critical proteins regulating HR. Therefore, when both repair mechanisms are being blocked by BRCA1/2 mutation and application of PARP-1 inhibitor, the cancer cells can no longer properly fix the DNA damage, and the cells will experience synthetic lethality (Woo et al., 2021). Furthermore,

the PARP-1 inhibitors were found to be sensitive in the cells that harbor other genomic mutations such as FANCC, ATM, NBS1, RAD51 and PTEN which are critical proteins involved in HR (Harris et al., 2018; Li et al., 2021; Woo et al., 2021).

1.5 Regulation of PARP-1 by ubiquitin-proteasome system

Ubiquitination is a protein post-translational modification and one of the key mechanisms for the regulation of PARP-1 functionally (Bricelj et al., 2021; Chauhan et al., 2016).

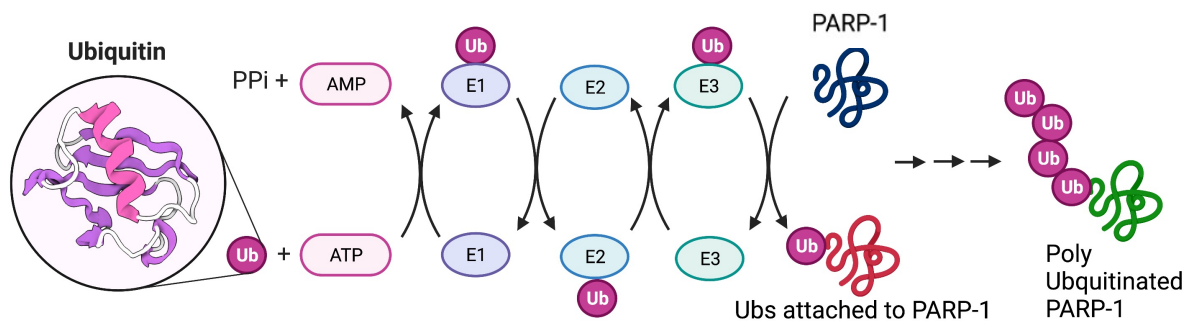
Ubiquitination requires three main steps, *i.e.*, activation, conjugation, and ligation. These three steps are regulated by E1, the ubiquitin-activating enzymes, E2, the ubiquitin-conjugating enzymes, and E3, the ubiquitin ligases respectively (Rogers et al., 2021; Skowron et al., 2021). In humans, only one member for the E1 ubiquitin activating enzyme has been identified, and around 40 members for the E2 conjugating enzymes. However, compared to E1 and E2s, E3 ligases belong to a larger protein family containing 500-1000 members in humans (Sui et al., 2020; Thomas et al., 2019). They are further categorized into four subfamilies including HECT, RING-finger, U-box, and RBR domain based on the catalytic domain that the E3 ligase possesses (Yang et al., 2017). Unlike E1 and E2, E3 ligases are responsible for conjugating the ubiquitin to specific proteins, which is the key step for substrate specificity. As one of the key regulatory mechanisms for PARP-1, PARP-1 undergoes ubiquitination by several E3 ligases, such as TRIP12, RNF144A, WWP2, Smurf2 and Iduna (Eslamloo et al., 2020; Fu et al., 2021; Kaluzinska et al., 2021; Kang et al., 2011; Zhang et al., 2020).

TRIP12 is a newly identified HECT and WWE domain containing E3 ligase for PARP-1. The TRIP12 knockdown can sensitize the cells to PARP inhibitor. Its functions in oncology were cancer type dependent, it can be oncogenic to promote the initiation and growth in some types of tumor, whereas it also inhibited cancer metastasis in a study using the MCF10A breast cancer

cells. (Kaluzinska et al., 2021). RNF144A is a tumor suppressor protein and can ubiquitinate PARP-1 via its residues 253-292 located at C-terminal region containing transmembrane domain. Recent studies have shown that it is being downregulated or even silenced in some types of breast cancers which led to an elevated level of PARP-1 protein stability (Eslamloo et al., 2020).

WWP2 is an E3 ligase that was recently discovered to ubiquitinate PARP-1. The interaction with PARP-1 is through WWP2 BRCT domain and the mutations in its lysine 418 and lysine 249 are critical for the regulation of PARP-1 binding (Zhang et al., 2020). Unlike TRIP12, RNF144A and WWP2, Smurf2 is a HECT domain containing E3 ligase for PARP-1, but it mainly monoubiquitinates PARP-1 by interacting with the PARP-1 residue lysine 18 (Fu et al., 2021). The interactome analysis revealed that Smurf2 can facilitate PARP-1 activity in DNA lesions, and potentiate PARP-1 associated bioactivities. (Fu et al., 2021). Iduna is an E3 ligase for many DDR associated proteins such as XRCC1, DNA ligase III, KU70 and PARP-1. The binding between Iduna and PARP-1 is highly dependent on the presence of PAR and the integrity of Iduna RING domain. The depletion of PAR abolishes the binding between the Iduna RING domain and PARP-1, also the mutation at Y156A and R157A within the Iduna WWE domain will abolish the binding between Iduna and PAR, which also leads to abrogation of PARP-1 ubiquitination by Iduna (Kang et al., 2011; Thomas et al., 2019; Tschope et al., 2020).

1 Ubiquitination



2 Protein degradation

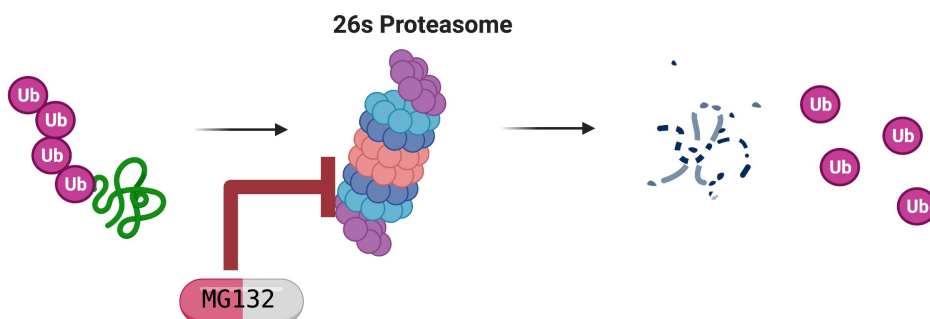


Figure 1.4 The ubiquitination-proteasome degradation pathway. Step 1, The specific target proteins, such as activated PARP-1, are ubiquitinated by E3 ligase to form a polyubiquitin chain. Step 2, The ubiquitin chain can be recognized by 26S proteasome. The proteasome will degrade both the polyubiquitin chain and the associated target proteins into amino acids. MG132 can block the activity of 26S proteasome and prevent the degradation of ubiquitinated proteins (Bricelj et al., 2021; Chauhan et al., 2016; Skowron et al., 2021; Valenzuela et al., 2020).

The common feature of these PARP-1 E3s is to modulate PARP-1 activity and stability through ubiquitination (Crawford et al., 2020; He et al., 2015; Rogers et al., 2021). General PARP-1 ubiquitination involves PARP-1 specific E3 ligase mediated transferring of ubiquitin molecules to the ϵ -amino group of the lysine residues to form polyubiquitinated PARP-1 (Figure 1.4). The polyubiquitin chains can be recognized by 26S proteasomes and the ubiquitinated proteins will subsequently be degraded by the proteasome (Bricelj et al., 2021; Chauhan et al.,

2016). Though a full scope of PARP-1 associated ubiquitination is not clear, it has shown prominent impact on the regulation of PARP-1 in many biological and pathological aspects.

1.6 The role of HUWE1 in DNA damage

HUWE1, also known as Mule, ARF-BP1, E3 Histone, URB1, HECTH9 and LASU1, is an E3 ligase which covalently attaches ubiquitin to many cellular substrate proteins including p53, cMyc, histones and β -catenin, to regulate their function and protein stability (Bartoli-Leonard et al., 2021; Han et al., 2021; Leung et al., 2019; Su et al., 2021; Zhao et al., 2021).

Human HUWE1 is encoded by the HUWE1 gene located on chromosome Xp11.22. The protein is identified as a multifaceted HECT domain-containing ubiquitin E3 ligase. The human HUWE1 is a multi-domain protein comprised of 4374 residues, which contains a N-terminal ARM domain with unknown function, followed by UBA and WWE domains, a BH3 domain and a C-terminal catalytic HECT domain (Figure 1.5). (Aprigliano et al., 2021; Clements et al., 2020).

The HUWE1 WWE domain is a globular domain containing two conserved tryptophan (W) and one glutamate (E) residues, and the total size is around 80 residues (Figure 1.5). It is also found in various other HECT and RING type E3 ligases. The WWE domain is believed to mediate specific protein-protein interaction, also recently it was characterized as a structural motif to recognize the ADP-ribose unit of PAR polymers. This implies that HUWE1 is a “reader” protein to recognize and bind PAR chains. The HUWE1 HECT domain contains two globular subdomains, the N-terminal subdomain and a C-terminal subdomain, connected by a flexible linker (Figure 1.5). An E2 binding site is located at the N-terminal subdomain, whereas the C-terminal subdomain contains a catalytic cysteine residue, responsible for the transfer of ubiquitin

molecules from E2 to E3 ligases. The cysteine residue functions to catalyze the formation of the ubiquitin-thioester intermediate (Jang et al., 2021; Kunz et al., 2020; Serebrovskaya et al., 2020).

HUWE1 has been reported to participate in the DNA damage repair process through ubiquitination. Mandemaker *et al.* have reported that cellular DNA damage was dramatically increased in HUWE1 knockdown cells under UV light treatment (Mandemaker et al., 2017). In addition, HUWE1 has been shown to regulate stability of several protein involved in DDR including histone H1, BRCA1 and proliferating cell nuclear antigen (PCNA) (Wenmaekers et al., 2021). Therefore, understanding the role and mechanism of HUWE1 in DDR signaling can provide insight to the regulation of DDR processes in the cell.

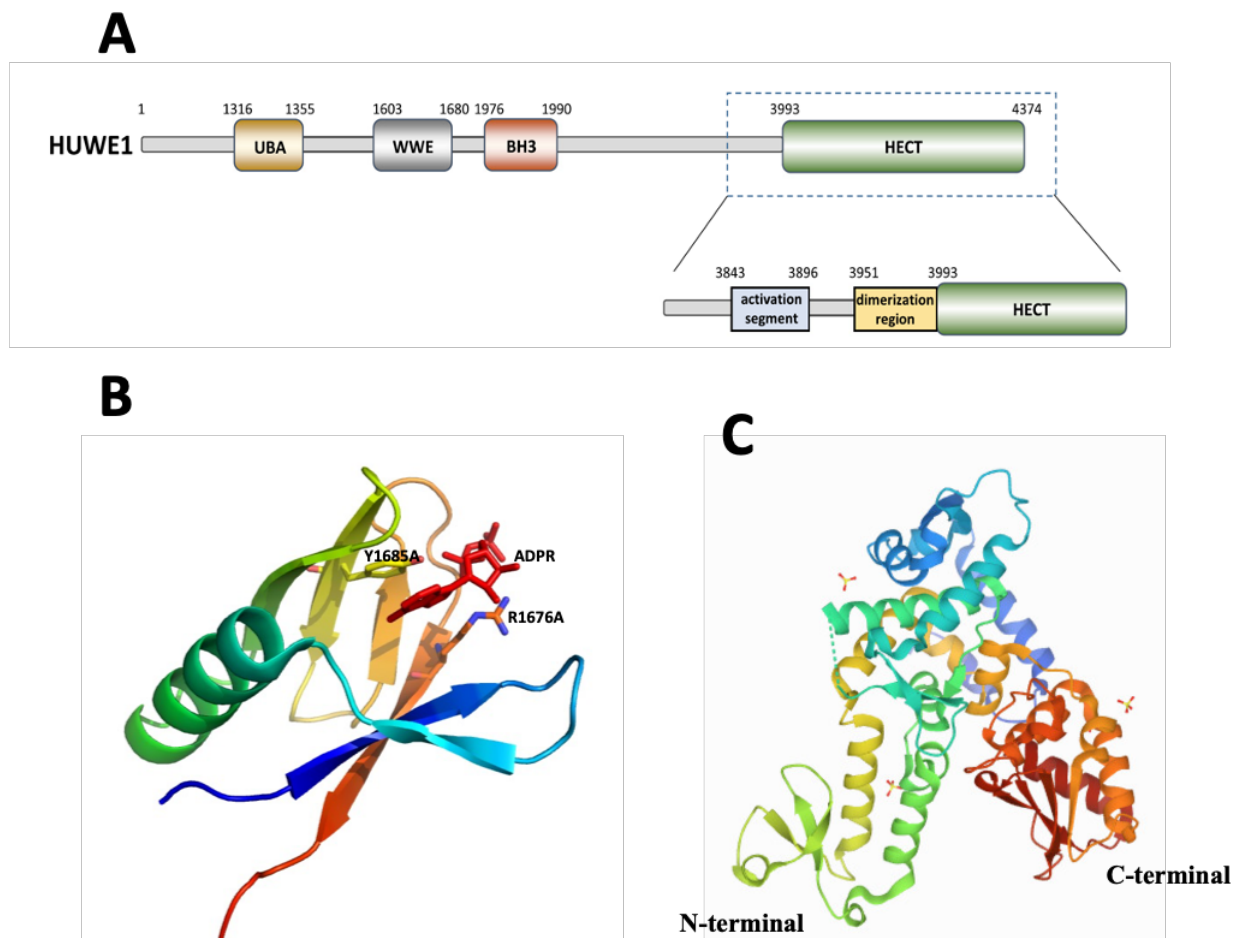


Figure 1.5 Schematic diagram of the structural domains of HUWE1.

A, HUWE1 contains four distinct functional domains: the UBA domain, the WWE domain followed by the BH3 domain, and lastly the HECT domain. Adapted from (Kao et al. 2012). B, Crystal structure of human HUWE1 WWE domain in complex with ADPR (PDB 6PFL). C, Crystal structure of HUWE1 HECT domain (PDB 3H1D). Ribbon diagrams of HUWE1 WWE domain and HECT domain were prepared using PYMOL.

1.7 Research Rationale

The cell has developed many mechanisms to repair DNA lesions, such as base excision repair, nucleotide excision repair, mismatch repair, homologous recombination and non-homologous end joining (He et al., 2015). In these repair pathways, PARP-1, a DDR associated

protein, plays an important role in recruiting other DDR regulatory proteins and controlling their activation by PAR synthesis and PARylation (Houl et al., 2019). Although PARP-1 is not mandatory in HR specific DNA damage repair, it participates in HR pathway protein signaling to facilitate DDR. For example, PARP-1 can protect HR from the interferences by Ku70 and Ligase IV in chicken embryonic cells (Matyas, Hasko et al. 2020). Other than HR, PARP-1 is a key protein involved in various DNA repair pathways and is critical for cell survival under DNA damage (Mandemaker et al., 2017; Matyas et al., 2020). Many PARP-1 inhibitors, such as Olaparib and Talazoparib, have been approved to treat cancers containing mutations in BRCA1/2, PALB2, and other HR associated DDR proteins (Cui et al., 2021). PARP-1 inhibitors have shown clinical efficacy by causing synthetic lethality to cancers with a defective HR pathway. On the other hand, the overreaction of PARP-1 can cause elevated PAR synthesis, which would lead to cell death called parthanatos (Koehler et al., 2021). Parthanatos is closely associated with many diseases such as stroke and ischemia injury (Nielsen et al., 2020). Therefore, understanding the mechanisms that regulate PARP-1 activity and stability is critical to elucidate the cellular signaling pathways required for cell survival and homeostasis.

Several E3 ligases such as UHRF1, RNF144A, WWP2, Smurf2 and Iduna have been shown to modulate PARP-1's stability and activity by ubiquitination (Eslamloo et al., 2020; Fu et al., 2021; Kaluzinska et al., 2021; Kang et al., 2011; Zhang et al., 2020). HUWE1 is a HECT domain containing E3 ligase which has been reported to ubiquitinate several DDR associated proteins such as p53, PCNA, γ H2AX and several translesion polymerases (Caballero-Solares et al. 2020). HUWE1 can also regulate many critical targets from both tumor suppressors to oncogenic proteins. Therefore, the role of HUWE1 in tumorigenesis is controversial and dependent on cellular context and cancer cell types (Yang et al., 2017). Dysregulation of HUWE1

is generally regarded as a signal for poor tumor prognosis (Aprigliano et al., 2021; Wenmaekers et al., 2021).

Taken together, though the clinical importance of HUWE1 and PARP-1 have been well appreciated, the functional correlation between these two proteins is still a mystery at the molecular level. Moreover, though both HUWE1 and PARP-1 are involved in DDR signaling, the functional relationship between them in DDR has not been well and fully characterized.

Human bone osteosarcoma epithelial cell (U2OS) and human embryonic kidney 293 cells with SV40 large T antigen (293T) are two of the most commonly used cell lines in biological study. U2OS was originated from a moderately differentiated osteosarcoma patient and was one of the first cancer cell lines isolated. It has very low number of chromosomal numerical variations and normal functional p53 compared to other sarcoma cells which make this cell line suitable to study the DNA damage repair pathway (Lauvrak et al., 2013). 293T is a cancer cell line used widely in cell biology research, which is the ideal host for plasmid transfection and ectopic protein expression. The SV40 large T antigen in the 293T cells allows the multiplication of transfected plasmids more steadily and efficiently (Thomas and Smart, 2005). Therefore, these two cell lines were used in my research experiments.

1.8 research aims

Aim 1 - To characterize the interaction between HUWE1 and PAR, and between HUWE1 and PARP-1

The major role of PARP-1 is to synthesize PAR chains and subsequently PARylate other proteins. Additionally, PARP-1 can also modify itself by auto-PARylation. It has been reported that the RING-type E3 ubiquitin ligase Iduna WWE domain recognizes the PAR chain and is

responsible for PARylation-dependent ubiquitination (Wang et al., 2012). Also, mutation in the WWE domain abolished the ubiquitination by RNF146 (DaRosa et al., 2015; De Vos et al., 2014). HUWE1 contains a WWE domain, which could interact with the ADP-Ribose group of PAR. Therefore, I hypothesize that HUWE1 can interact with both PAR and PARP-1, as PARP-1 can mediate self-PARylation.

To test this hypothesis, I will overexpress FLAG-HUWE1 within 293T cells and induce DNA damage to activate PARP-1. Then I will perform co-immunoprecipitation experiments to pull down PAR, PARP-1 and FLAG to study the interaction of FLAG-tagged HUWE1 with PAR/PARP-1.

Aim 2 - To characterize HUWE1 as a E3 ligase for PARP-1.

The major role of E3 ligases are to ubiquitinate specific target proteins (Clements et al., 2020). Therefore, it is hypothesized that HUWE1, as an E3 ligase, ubiquitinates PARP-1. In order to examine this hypothesis, I will treat the cells with MG132 in addition to etoposide to induce DNA damage and PARP1 activation. MG132 is a 26S proteasome inhibitor which prevents degradation of poly-ubiquitinated proteins by the proteasome. I hypothesize that by applying the MG132, PARP-1 ubiquitination level will increase as the ubiquitinated PARP-1 cannot be degraded. Ubiquitinated PARP-1 could be detected in the HUWE1 overexpressed 293T cells, more abundant than that of the control 293T cells.

Aim 3 - Profile HUWE1 and PARP-1 responses under DNA damage.

If HUWE1 indeed regulates PARP-1 stability, it is expected to observe a reverse relationship between HUWE1 and PARP-1 protein levels. To test the hypothesis, I will induce U2OS cells by DNA damaging agent etoposide in different time intervals. Then I will collect

cytosolic and nuclear proteins and perform western blot to detect the protein changes in different time course and at different subcellular compartments.

Meanwhile, I will use two different HUWE1 shRNAs (F6 and G10) with an empty vector control (GIPZ) to infect U2OS cells to knock down the HUWE1 mRNA level and HUWE1 protein synthesis. If HUWE1 indeed regulates PARP1 stability, it is expected that the ShRNA treated cells will show a significant increase of PARP-1 protein level. IF will be performed to visualize the PARP-1 signals from the ShRNA treated cells to measure the cellular PARP-1 levels compared to the control cells.

PARP-1 has been a critical drug target in many cancer cells with different genetic alterations. The systematic study of the impact of HUWE1 on PARP-1 will provide a new insight in cancer studies and an important potential approach to elucidate further mechanistic understandings to target PARP-1 in both basic research level and clinical aspect.

Chapter 2 : Material and methods

2.1 Cell Culture

Human Embryonic Kidney cells (HEK 293T) cells were kindly provided as a gift from Dr. Peng's laboratory (York University) and were cultured in DMEM media (Life Technologies Cat# 11995065) supplemented with 10% fetal bovine serum (FBS, Gibco Cat#: 26140079) and 1% penicillin-streptomycin (P/S, Life Technologies Cat# 15070063). HEK 293T with FLAG-tagged HUWE1 stably overexpressed cells were kindly provided as gift from Dr. Mak's laboratory (University of Toronto) and were cultured in DMEM media supplemented with 10% FBS, 1% P/S, and 120ug/ml of geneticin (G418, Gibco Cat#10131-035) to retain cells expressing the selected plasmids (Dominguez-Brauer et al. 2017). Osteosarcoma (U2OS) cells were kindly provided as a gift from Dr. Benchimol's laboratory (York University) and were cultured in McCoy's 5A modified medium (Life Technologies Cat# 16600082), and supplemented with 10% FBS and 1% P/S.

2.2 Plasmid transformation and amplification

All the plasmids used in the following experiment were transformed into DH5a *E. coli*. pCI-neo-HUWE1 R1676A and Y1658A plasmid containing DH5a were amplified in 100ug/ml ampicillin containing LB overnight at 30 °C. PAX2, VSVG, pGIPZ, pGIPZ shHUWE1-F6 and shHUWE1- G10 plasmid containing DH5a were amplified in 100ug/ml ampicillin containing LB for overnight at 37°C.

2.3 Plasmid transfection to overexpress HUWE1 and WWE mutants

The plasmids pCI-neo-HUWE1 R1676A and Y1658A mutants were used to overexpress HUWE1 WWE domain mutated proteins in 293T cells. PolyJet transfection Kit (SignaGen Cat#

SL100688) was used for the plasmid transfection to 293T cells according to manufacturer's protocol. For a typical 10×10^6 cells, 25 μ g of RA and YA, and 50 μ l PolyJet were used for the transfection. The cells were harvested after 48 h of transfection.

2.4 Cell treatment (DNA damage induction, PARP-1 inhibition, and MG 132 treatment)

For the IP experiment, DNA damage (2-4 hours) was required to activate PARP-1 and subsequently examine the interaction between HUWE1 and PARP-1. In order to induce DNA damage during the experiment, a final concentration of 10 μ M etoposide (Sigma Cat# E1383) was used to treat cells with various time intervals as described in the results. In the experiment to elucidate the role of PAR in PARP-1 and HUWE1 binding, PARP-1 inhibitor PJ34 10 μ M (Sigma Cat# 344458-15-7) was used to treat the cells to inhibit PARP-1 activity, and to decrease the synthesis of PAR chains along with etoposide treatment. To check whether HUWE1 is a E3 ligase for PARP-1, 5 μ g/ml MG132 (Calbiochem Cat# 474787-10MG) was used along with etoposide treatment to prevent the ubiquitinated PARP-1 from being degraded by the 26S proteasome.

2.5 Co-immunoprecipitation

Co-immunoprecipitation was carried out to study cellular interactions in 293T cells, specifically, the interaction between HUWE1 and PAR, HUWE1 and PARP-1. Generally, 20×10^6 cells were lysed with 1ml of IP lysis buffer (25mM Tris•HCl pH 7.4, 150mM NaCl, 1% NP-40, 1mM EDTA, 5% Glycerol) supplemented with protease inhibitor (100 μ M Phenylmethane Sulfonyl Fluoride (PMSF), 1 μ M Benzamidine Hydrochloride, 1X Protease Cocktail Inhibitor

(PI)) (Roche Cat# 5056489001) and 1 μ M ADP-HPD PARG inhibitor (Calbiochem Cat#118415-60UG). 10% of total lysate were used as the input sample for western blot.

To perform co-immunoprecipitation, 4 μ l of primary antibody were added into 2mg of total protein lysate and incubated for 3 hours at 4°C on the rocker. Then 50 μ l protein A/G beads (Santa Cruz Cat# sc-2003) were added into the lysate directly and incubated for another 1 hour at 4°C on the rocker. After a totally 4 hours incubation, the supernatant was removed by centrifuge (4,000g for 2 minutes) and collecting the A/G beads. The precipitant immobilized on protein A/G beads were washed 5 times, 10 minutes for each time, with 1ml IP lysis buffer in the rocker at 4°C. The precipitant was denatured with 5X SDS-loading buffer at 100°C for 10 minutes and subject to immunoblotting.

2.6 Fractionation preparation

Cell fractionation was performed using a commercial fractionation kit according to manufacturer's instruction (Thermo Cat#78833). Approximately 10 million cells were lysed in 200 μ l of NE-PER cytoplasmic extraction buffer with additional 100 μ M PMSF and protease inhibitors as listed in 2.5. Samples were incubated for 10 minutes at 4°C. Then cytoplasmic extraction buffer II was added to the sample and incubated on ice for 1 minute. The sample was centrifuged at 20,000 g for 15 minutes to pellet the nuclear fraction, and the supernatant containing the cytoplasmic proteins. To solubilize the nuclear protein, the nuclear fraction was dissolved with RIPA buffer (25mM Tris•HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS), and incubated for 1 hour at 4°C with intermittent vigorous vortexing.

Then the sample spun down at 20,000 g for 15 minutes and to collect nuclear supernatant. The nuclear fractionation was subject to immunoblotting analysis.

2.7 ShRNA knockdown HUWE1

The pGIPZ shHUWE1-F6 (F6) and shHUWE1-G10 (G10) plasmids were gifts from Dr.Mak's laboratory. (Inoue et al., 2013) The shRNAs were inserted between mir5' to mir3' as shown in Figure 2.1. ShRNA Huwe1 lentiviral vectors (pGIPZ system) were obtained from Open Biosystems, catalog no. RHS4531-NM_031407; clone ID: V2LHS_110969 (F6) and V2LHS_262858 (G10).

PAX2, VSVG, pGIPZ, F6 and G10 plasmids were transfected in 293T cells to produce lentivirus containing pGIPZ, F6 and G10 respectively. For the lentiviral transfection, one typical T175 flask would need 10 µg VSVG, 20 µg PAX2, 10 µg pGIPZ , F6 or G10 with 80µl Polyjet. Lentiviral supernatants were harvested 24 hours post transfection and centrifuge at 3,000 RPM for 5 minutes. The supernatants containing the lentivirus particles were filtered through a 0.22µm syringe filter and stored at 4°C for short time or -80°C for long term storage.

Plasmid Maps

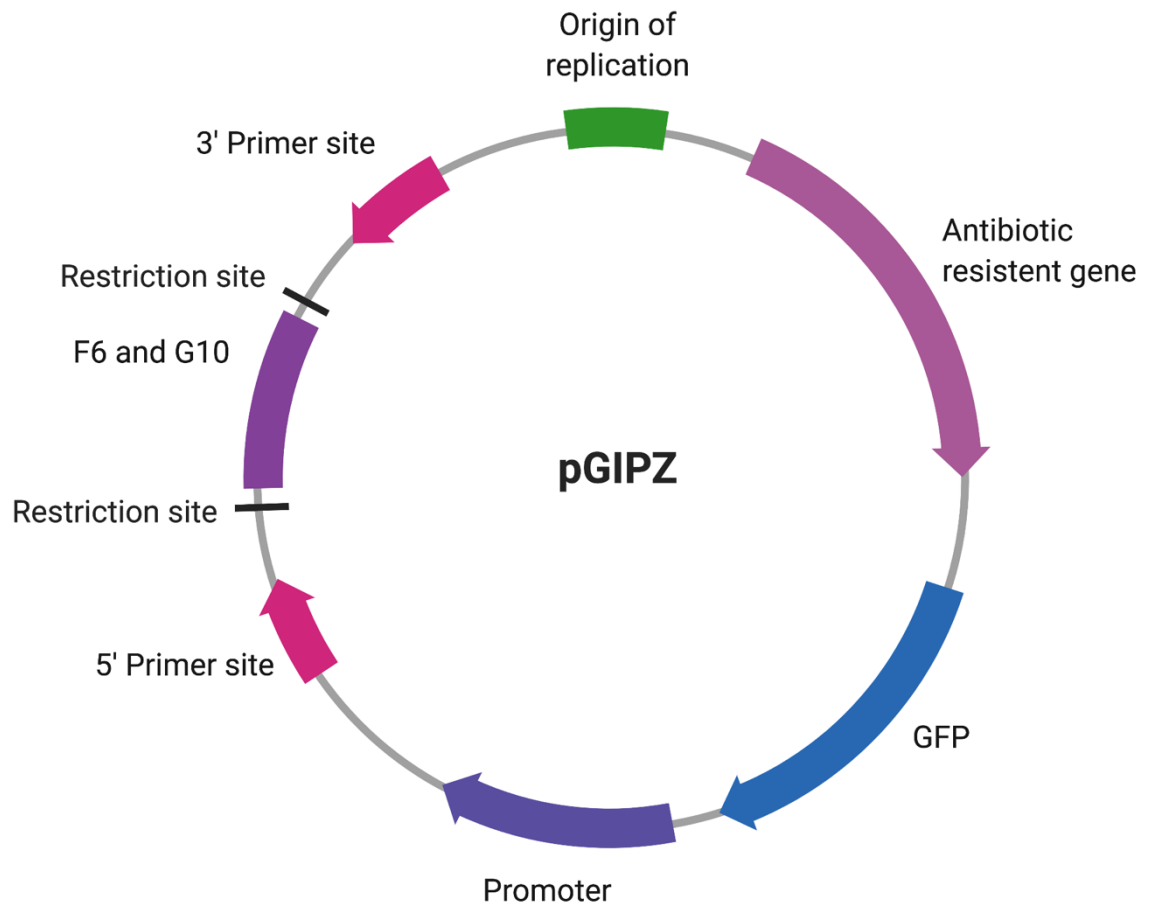


Figure 2.1 pGIPZ shRNA vector expression system used for shHUWE1-G10 and shHUWE1-F6 stably knocked down cell lines.

For lentiviral transduction of U2OS cells, U2OS cells were incubated with McCoy's complete media containing lentivirus solution (mixed with 1:1) along with 8µg/ml polybrene for 48 hours. The media was replaced with media supplemented with 4mg/ml Puromycin. After 24 hours incubation with puromycin, the U2OS cells were imaged by EVOS 5000M microscopy. If over 99% of total cells showed GFP signals because the shRNA plasmids contained GFP, the cells were used for shRNA based HUWE1 knockdown experiments.

2.8 Detection of HUWE1 and PARP1 mRNA expression using qPCR

This experiment was used to detect the mRNA levels of HUWE1 and PARP-1 in U2OS cells infected with pGIZP vector control, shHUWE1 F6 or G10 lentivirus. The entire procedure includes RNA isolation from each group of U2OS cells, preparation of cDNA from RNA, and carrying out qPCR using cDNA. The detailed methods were described below.

2.8.1 RNA isolation

U2OS cells (~ 5 x 10⁶) were lysed in 1ml of TRIZOL Reagent for each plate. Chloroform (0.2 ml) was added to each 1ml of TRIZOL Reagent immediately. Samples were vortexed vigorously for 15 seconds and incubated at room temperature for 2 to 3 minutes. After that, the samples were centrifuged at 12,000 x g for 15 minutes at 4°C. Following centrifugation, upper aqueous phase was transferred into a fresh tube, mixed with 0.5 ml of isopropyl alcohol, incubated at room temperature for 10 minutes and then centrifuged at 12,000 x g for 10 minutes at 4°C. Supernatant was discarded and the RNA pellets were washed two times with 75%

ethanol. RNA pellets were air-dried for several minutes and then dissolved in 0.1 ml of DEPC-treated water. The RNA concentration was measured by Thermo NANODROP 2000 using the “nucleic acid measure” option. The 260/280 ratio was between 1.8-1.86 for a pure RNA isolation.

2.8.2 cDNA preparation

mRNA was reverse transcribed to cDNA by High-Capacity-RNA to cDNA Kit (Thermo, cat# 4387406). 2µg mRNA was used for each 20µl reaction. The reaction was set up as shown in Table 2.1. The reaction mix was performed in Bio-RAD T100 Thermal Cycler. The samples were incubated at 37°C for 60 minutes, and then the reaction was stopped by heating the samples up to 95°C for 5 minutes and hold at 4°C. The cDNA sample is ready for use in real-time PCR applications or long-term storage in a freezer (−25°C to −15°C).

Table 2.1 The reaction mix for cDNA synthesis

Component	Volume per reaction	
	+RT reaction	−RT control
2X RT Buffer Mix	10.0 µL	10.0 µL
20X RT Enzyme Mix	1.0 µL	—
RNA sample	up to 9 µL	up to 9 µL
Nuclease-free H ₂ O	Q.S. ^[1] to 20 µL	Q.S. ^[1] to 20 µL
Total per reaction	20.0 µL	20.0 µL

2.8.3 qPCR reaction

To detect the mRNA levels of HUWE1 and PARP-1, qPCR reactions were performed according to manufacturer’s instruction using SsoFast EvaGreen kit (Bio-rad, Cat# 1725200). To make complete qPCR reaction mix, 2µl of diluted cDNA (1:3), 5µl EvaGreen mix, 0.5µl forward and reverse primers and 2µl DEPC water were added to make full volume into 10µl. 40 cycles were performed during qPCR by Thermo Rotor Gene Q, 95°C for 30 seconds for predenaturation

phase, 95°C for 10 seconds for primer extension phase and 60°C for 15 seconds for cDNA synthesis phase. The threshold was set to 0.15 for data analysis.

Table 2.2 Primers used in the RT-qPCR reaction

	Forward sequence	Reverse sequence
PARP-1 primers 1	GGCGATCTTGGACCGAGTAG	GTAATAGGCATCGCTCTTGAAG
PARP-1 primers 2	CCAAGCCAGTTCAGGACCTCAT	GGATCTGCCTTTTGCTCAGCTTC
HUWE1 primers 1	CAAACCTACATCACTCGTCTGGG	AGTCTCTGCAACATTCTGCAA
HUWE1 primers 2	CGGCTTTTCCTGAAGAAGGGAC	GTTTCAAAGGCTTCAGAGCAGC
HUWE1 primers 3	CCAGAAGTTCTTCTTGAGGGTAC	GCCTAAACCGGAGGAACC
GAPDH primers	AAGGTCATCCCTGAGCTGAAC	ACGCCTGCTTCACCACCTTCT

2.9 Immunoblotting

The cell pellets were lysed in 100µl of RIPA buffer for 30 minutes on ice supplemented with PMSF and protease inhibitors. The lysates were centrifuged at 20,000 g for 15 minutes to collect supernatants. Pierce BCA assay (Thermo, Cat #23221) was performed to detect protein concentration. 50-100µg samples were used for protein gel electrophoresis with 4%-12% pre-casting gels (Thermo, Cat #NP-0321). The protein samples were run at 115 V for 90 minutes at room temperature. The protein transfer was performed with 0.45µM PVDF transfer membranes

at 30V for 16 hours at 4°C. After the transfer is complete, PVDF membrane were blocked in 5% milk in TBST for 2 hours and followed by another blocking in 5% BSA in TBST for 1 hour at room temperature. Then the appropriate primary antibodies diluted in 1% BSA-TBST buffer were used to incubate with the membrane overnight at 4°C. The membranes were washed with TBST four times, 15 minutes for each wash. The secondary antibodies conjugated with HRP diluted in 1% milk-TBST buffer were used to blot the membrane for 1 hour at room temperature. After secondary antibody incubation, the membranes were washed with TBST for another four times, 15 minutes for each wash, and incubate with ECL (Perkin Elmer Cat #NEL105001EA) for a few seconds. Micro Chemiluminescence instrument was used to image the PVDF membranes.

2.10 Immunofluorescence (IF) assay

The IF assay was used for imaging intracellular protein signals. 50,000 U2OS cells were seeded on the coverslip in 12 well plates in complete McCoy 5A media. 4% methanol free PFA diluted in PBS were used to fix the cells at room temperature for 30 minutes. The PFA treated cells were incubated with 0.3% Tx-100 for 10 minutes. Blocking buffer (Thermo Cat #B10710) 0.5ml per well were used to block the cells for 1 hour at room temperature without any dilution.

Primary (anti-PARP-1, anti-BRCA1, anti-53BP1 and anti- γ H2AX) and secondary antibodies were all diluted in 1:200 dilution in blocking buffer. Cells were incubated with primary antibodies for 1 hour at room temperature and rinsed with TBST 3 times, 5 to 10 minutes for each time. The cells were then incubated with the secondary antibodies for 30 minutes at room temperature and rinsed with TBST 3 times, 5 to 10 minutes each time, at room temperature. Air dry the cells and then add the antifade-DAPI containing-hardening mounting oil (Invitrogen,

Cat # P36983) to seal it for further use. The slides were stored at 4°C avoiding light. After the images were obtained by Zeiss Spinning Disk Confocal Microscopy with the same settings in between different treatment groups, Image J was used to select the GFP positive cells cross the DAPI signal, then the protein signal intensities were collected from all the treated groups and the DMSO treated wild type VE group protein signals were normalized to one. Around 100-200 cells were counted at 5-10 different sample areas per treatment groups. The data was transferred into Graphpad V8.0 for analysis and graphing.

2.11 Cell titer Glo 2.0 cytotoxicity assay

To detect the impact of HUWE1 KD on cell survival, Cell titer Glo 2.0 (Promega Cat #G9243) was used to detect cellular ATP amount which is proportional to cell survival. 10,000 cells were seeded in each well in 96-well plate. Cells were treated with 10 μ M etoposide to induce DNA damage. The cell titer Glo was diluted in PBS (1:1) and added to each well and cells were shaken at 300 rpm for 10 minutes avoiding light. Then luminance signal was measured at 528/20 wavelength in the Synergy H4 Hybrid reader. The etoposide and AZD working concentrations were optimized by a dose-response curve.

2.12 Statistics

All the statistical analysis were performed by Graphpad software. The *p* values were calculated using ANOVA and multiple comparison test. Three biological replicates were performed in qRT-PCR reactions and Western blot for analysis. For IF analysis, a total of 100 – 200 cells were counted from three biological repeats for quantifying 53BP1, BRCA1 and PARP-1 signals.

Table 2.3 List of antibodies and associated dilution preparation

<u>Antibody Name</u>	<u>Manufacturer</u>	<u>Catalogue No.</u>	<u>Species</u>	<u>Dilution</u>	<u>Dilution in TBST</u>
HUWE1	Bethyl	A300-486A-M	Rabbit	1:1000 μ l	5% Milk
gH2AX(Ser139)	CST	80312	Mouse	1:1000 μ l	5% BSA
PARP-1	CST	9542S	Rabbit	1:1000 μ l	5% BSA
PARP-1 (HC2R8)	Invitrogen	14-6667-82	Mouse	1:1000 μ l	5% BSA
PAR (E6F6A)	CST	83732S	Rabbit	1:1000 μ l	5% BSA
Anti-PAR (Ab-1) (10H)	Trevigen	4335-MC-100	Mouse	1:1000 μ l	5% BSA
GAPDH	Santa Cruz	sc-47724	Mouse	1:5000 μ l	5% BSA
Lamin A/C	Santa Cruz	sc-376248	Mouse	1:1000 μ l	5% Milk
Lamin B2 (D8P3U)	CST	12255	Rabbit	1:1000 μ l	5% BSA
Phospho-NBS1 (Ser343)	CST	3001	Rabbit	1:1000 μ l	5% BSA
Ubiquitin (P4D1)	Santa Cruz	sc-8017	Mouse	1:1000 μ l	5% BSA
Phospho-ATM (Ser1981) (D6H9)	CST	5883	Rabbit	1:1000 μ l	5% BSA
Phospho-ATM (Ser1981) C.70.6	Invitrogen	MA5-15185	Mouse	1:1000 μ l	5% BSA
Anti-PAR (Ab-1) (10H)	Millipore/Calbiochem	AM80-100UG	Mouse	1:1000 μ l	5% BSA
H3	CST	14269	Mouse	1:1000 μ l	5% BSA
H2A-X	CST	2595	Rabbit	1:1000 μ l	5% BSA
Alexa 555 (IF)	Invitrogen	A32732	Rabbit	1:1000 μ l	5% BSA
Anti- β -Actin	Sigma	5441	Mouse	1:5000 μ l	5% BSA
Anti-Rabbit-HRP	Jackson Immuno	711-035-152	Donkey	1:5000 μ l	1% Milk
Anti-Mouse-HRP	Jackson Immuno	715-035-150	Donkey	1:5000 μ l	1% Milk
Anti-FLAG M2	Sigma	F1804	Mouse	1:1000 μ l	5% BSA
DAPI	Invitrogen	R37606	Drops	2 drops/ml	N/A
* Bovine Serum Albumin (BSA)					

Chapter 3 : Results

3.1 HUWE1 could directly interact with PAR

WWE domains predominantly exist in two classes of proteins, the proteins associated with ubiquitination and Poly-ADP ribosylation. Additionally, a direct binding between PAR and the WWE domain containing E3 ligase Iduna has been reported (Eslamloo et al., 2020; Fu et al., 2021; Kaluzinska et al., 2021; Kang et al., 2011; Zhang et al., 2020), which revealed that WWE domain functions as a PAR recognition motif. Another independent experiment also suggested that HECT domain containing E3 ligase TRIP12 can interact with PAR via its WWE domain (Eslamloo, Caballero-Solares et al. 2020, Zhang, You et al. 2020). Considering E3 ligase HUWE1 shared many similar features with Iduna, I hypothesized that HUWE1 can interact with PAR via its WWE domain.

To test this hypothesis, the plasmids containing either FLAG-tagged wild type HUWE1 (FLAG-WT) or two HUWE1 WWE domain mutants, R1676A and Y1658A (FLAG- RA and FLAG-YA), were transfected into 293T cells to allow the overexpression of FLAG-WT, FLAG-RA and FLAG-YA within the cells (Figure 3.1 A). R1676 and Y1658 are two critical residues within the HUWE1 WWE domain, and their mutation affects the structural folding of the WWE domain or its binding with ADP-ribose biochemically (unpublished data). As PARylation activity of PARP-1 could be greatly stimulated by DNA damage, etoposide was applied for four hours to induce DNA damage and to activate PARP-1 mediated PARylation. Indeed, etoposide treatment increased the cellular PAR, whereas the PJ-34, a PARP-1 inhibitor, decreased the PAR level (Figure 3.1.A).

To determine whether HUWE1 is a PAR-binding protein, co-immunoprecipitation was performed with the 293T cells overexpressed FLAG-tagged HUWE1. A PAR specific antibody was used to precipitate the cellular PAR chains. The PAR antibody could readily pull down both

PAR and FLAG- WT HUWE1, suggesting that HUWE1 is a PAR-binding protein. Notably, the PAR antibody could not pulldown FLAG-tagged WWE mutants of HUWE1(Figure 3.1.B), suggesting the integrity of the HUWE1 WWE domain was critical in modulating the binding between HUWE1 and PAR.

To further confirms the interaction between HUWE1 and PAR, a reciprocal immunoprecipitation was performed using FLAG specific antibody to pulldown the FLAG-tagged HUWE1 WT, and WWE1 domain mutants FLAG-RA and FLAG-YA (Figure 3.1.C). As expected, only the FLAG-WT HUWE1 was able to pulldown PAR, not the WWE1 domain mutants FLAG-RA or FLAG-YA. Taken together, HUWE1 and PAR could interact in the cell and the integrity of WWE domain is critical for this interaction.

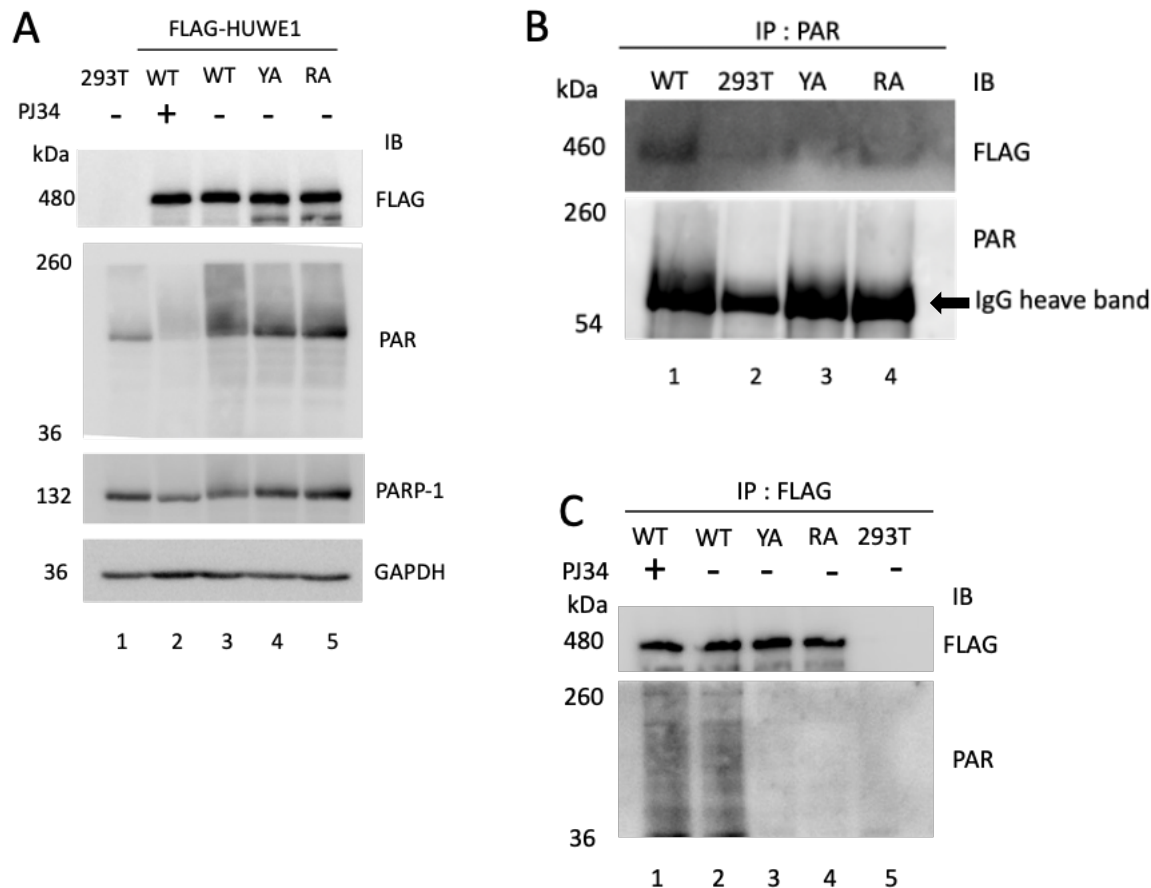


Figure 3.1 HUWE1 interacted with PAR via HUWE1 WWE domain. 293T cells were transfected with FLAG-tagged WT HUWE1 and two HUWE1 WWE domain mutants YA and RA. The cells were treated with 10 μ M etoposide with or without 10 μ M PJ34 for 4 hours. A. 293T cells total cell lysate was probed with various antibodies as indicated. B. WT HUWE1 interacted with PAR. Cellular PAR chains were immunoprecipitated with PAR antibody. Immunoprecipitants were blotted with FLAG and PAR antibodies. C. FLAG-HUWE1 and the mutants were immunoprecipitated with FLAG antibody. Immunoprecipitants were blotted with FLAG and PAR antibodies.

3.2 HUWE1 can interact with PARP-1 via WWE domain

The major role of PARP-1 is to synthesize PAR chains upon activation by DNA lesions and to PARylate the substrate proteins and itself (Cui et al., 2021). Since HUWE1 was capable of binding PAR, I hypothesized that the PAR chain would mediate the interaction between HUWE1 and PARP-1.

To test this hypothesis, 293T cells were used to overexpress FLAG-tagged HUWE1 WT, and WWE domain mutants RA and YA as described above (Figure 3.1.A). Consistent with the previous observation, PJ34 greatly inhibited the activity of PARP-1 and subsequently decreased overall production of PAR chains (Figure 3.2.A). PARP-1 antibody could immunoprecipitate PARP-1 protein, together with FLAG-tagged HUWE1 WT, but not YA and RA, suggesting that HUWE1 could interact with PARP-1 and the interaction was mediated through HUWE1 WWE domain. After PJ34 treatment, there was a decreased interaction between HUWE1 and PARP-1, suggesting PARylation of PARP-1 enhanced the interaction between HUWE1 and PARP-1 (Figure 3.1.B).

Furthermore, a reciprocal experiment was performed to pull down ectopically expressed HUWE1 WT, WWE domain mutants RA and YA using a FLAG antibody. As expected, PARP-1 could be readily pulled down by FLAG-tagged HUWE1 WT, but showed much weaker affinity for RA and YA, confirming the critical role of WWE1 domain in mediating the interaction between HUWE1 and PARP-1. PJ34 treatment decreased the interaction between HUWE1 and PARP-1, further confirming that the interaction between HUWE1 and PARP-1 could be promoted by PARylation of PARP-1 (Figure 3.1.C).

In addition, it was observed that the level of PARP-1 protein was decreased in the cells overexpressed with FLAG-tagged HUWE1 WT, compared to that of the 293T negative control,

YA and RA mutant groups. PJ34 treatment did not rescue HUWE1 WT mediated PARP-1 reduction (Figure 3.2). This result suggested that HUWE1 could play a role in the regulation of PARP-1 stability.

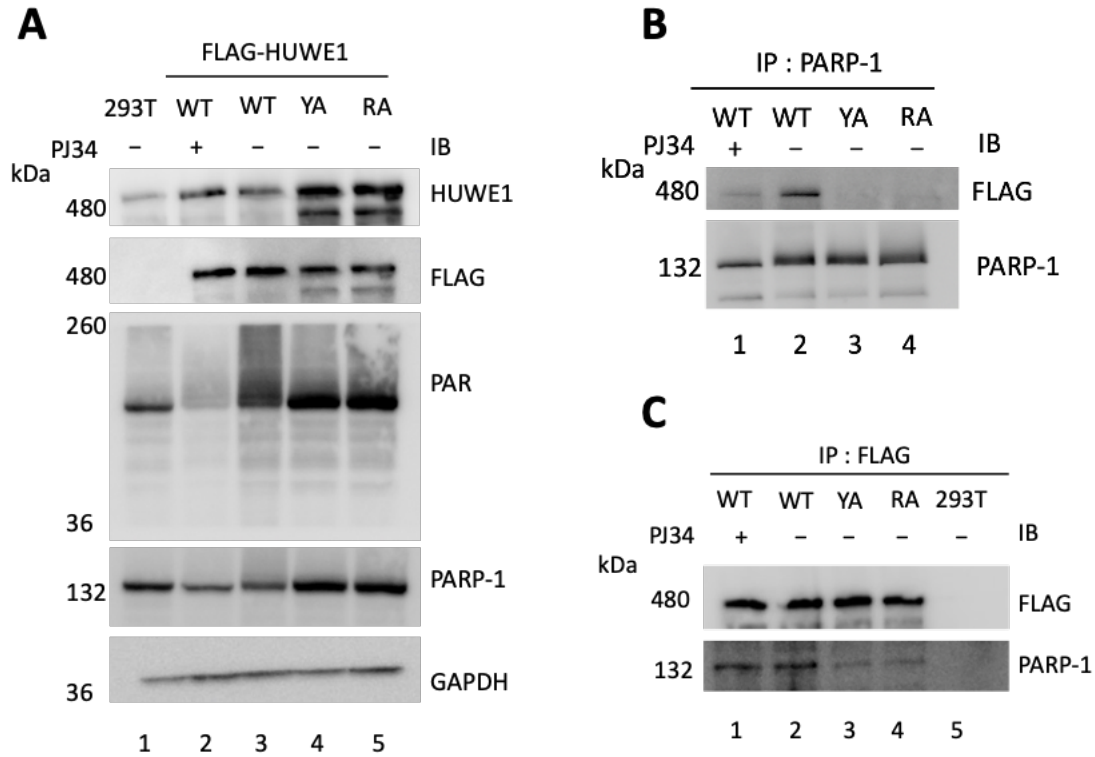


Figure 3.2 HUWE1 interacted with PARP-1 via HUWE1 WWE domain.

293T cells were transfected with FLAG-tagged wild type HUWE1 and two HUWE1 WWE domain mutants YA and RA. The cells were treated with 10 μ M etoposide with or without 10 μ M PJ34 for 4 hours. A, 293T cells total cell lysate probed with various antibodies as indicated. B, WT HUWE1 interacted with PARP-1. Cellular PARP-1 were immunoprecipitated with PARP-1 antibody. Immunoprecipitants were blotted with FLAG and PARP-1 antibodies. C, FLAG-HUWE1 and the mutants were immunoprecipitated with FLAG antibody. Immunoprecipitants were blotted with FLAG and PARP-1 antibodies.

3.3 HUWE1 is a E3 ligase for PARP-1 and mediates PARP-1 Ubiquitination

It was well established that PARP-1 ubiquitination facilitates PARP-1 degradation via 26S proteasome (Rogers et al., 2021; Skowron et al., 2021). As HUWE1 is an E3 ligase for many DDR associated proteins and interacted with PARP-1, I hypothesized that HUWE1 is a E3 ligase for PARP-1 and mediates PARP-1 ubiquitination.

To test this hypothesis, the proteasome inhibitor MG132 (10 μ M) was added to 293T cells with overexpressed FLAG-tagged HUWE1 WT, catalytically inactive mutant CA, or WWE domain mutants YA and RA, to enrich the ubiquitinated proteins in the cell. PARP-1 ubiquitination was detected by immunoblotting using ubiquitin antibody following an immunoprecipitation with PARP-1 antibody. As shown in Figure 3.3.A, PARP-1 ubiquitination was the most prominent in the 293T cells overexpressing FLAG-tagged HUWE1 WT, diminished in the 293T cells overexpressing the catalytically dead mutant CA or two WWE domain mutants RA and YA (Figure 3.3.B). This result suggested that HUWE1 is a PARP-1 E3 ligase and mediates PARP-1 ubiquitination. The functional integrity of both HUWE1 HECT domain and WWE domain are important for HUWE1 mediated PARP-1 ubiquitination and subsequent PARP-1 degradation by the 26S proteasome.

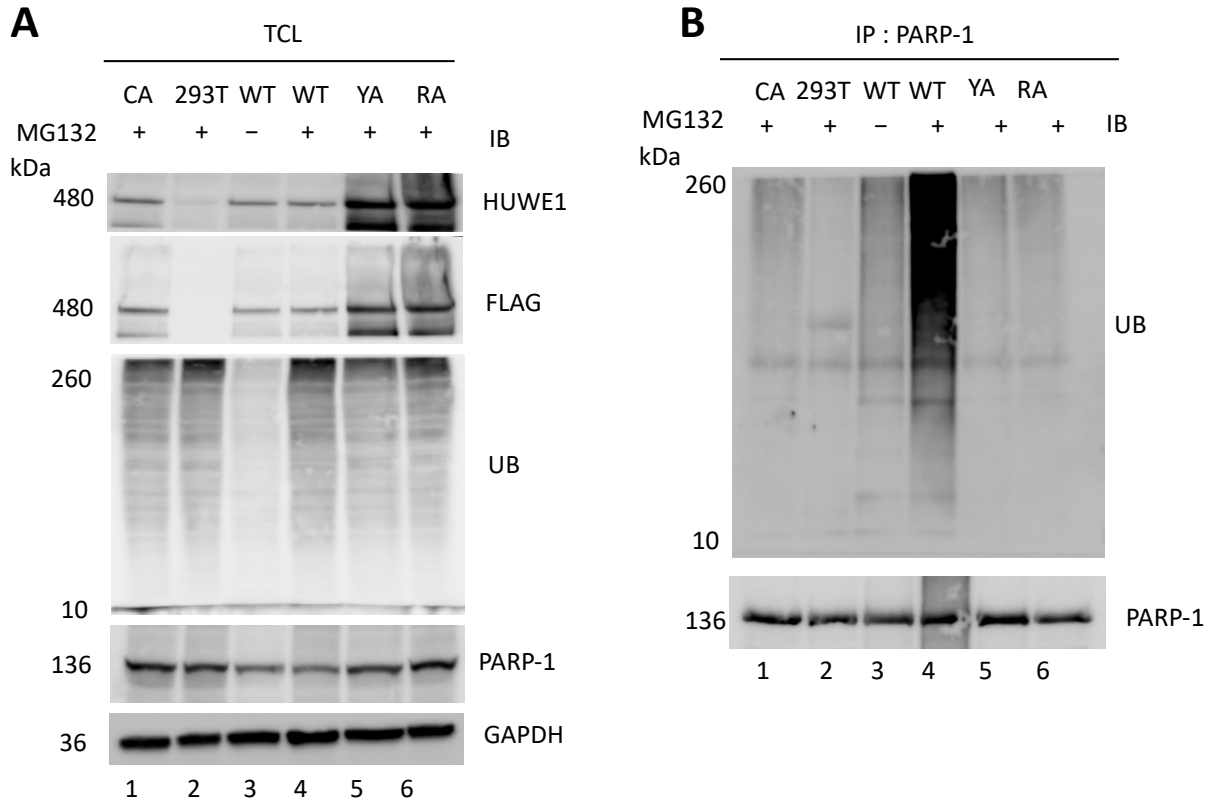


Figure 3.3 HUWE1 is a E3 ligase for PARP-1 and mediated PARP-1 ubiquitination. 293T cells were transfected with FLAG-tagged HUWE1 WT, catalytically inactive mutant CA, and two HUWE1 WWE domain mutants YA and RA. The cells were treated with 10 μ M etoposide with or without 10 μ M MG132 for 4 hours. A, 293T cells total cell lysate probed with various antibodies as indicated. B, HUWE1 WT ubiquitinated PARP-1. Cellular PARP-1 were immunoprecipitated with PARP-1 antibody. Immunoprecipitants were blotted with ubiquitin and PARP-1 antibodies.

3.4 HUWE1 knockdown increased the PARP-1 protein level in U2OS cells.

Previous experiments have suggested that overexpression of HUWE1 could decrease the cellular levels of PARP-1 (Figure 3.1.A, Figure 3.2.A and Figure 3.3.A). To investigate the role of HUWE1 in the regulation of PARP-1, I studied the effect of HUWE1 knockdown on PARP-1 in U2OS cells.

GFP expressing shRNA lentivirus system was used to stably knock down HUWE1 in the U2OS cells. This includes two separate steps, using 293T cells to prepare shRNA lentiviruses and followed by lentivirus transduction of U2OS cells. I first prepared the lentiviral particles for two GFP-expressing HUWE1 shRNA constructs, F6 and G10, and a GFP-expressing empty vector by the VSVG-PAX2 packaging lentiviral system in 293T cells. Stably knockdown HUWE1 was carried out in U2OS cells transduced with the purified lentiviral particles containing GFP-HUWE1 shRNA F6 and G10, or the control GFP-empty vector (VE). Following three days treatment with 3 μ g/ml puromycin to select for the cells stably expressing GFP-shRNA, GFP signal was monitored by fluorescence microscopy to ensure over 99% of the total cells express GFP.

To examine whether HUWE1 was knocked down in the U2OS cells stably expressing HUWE1 shRNA constructs F6 and G10, HUWE1 mRNA levels were evaluated using qRT-PCR. Three different HUWE1 primers targeting the N-terminal, central and C-terminal regions of HUWE1 cDNA were used to determine the mRNA levels of HUWE1 and GAPDH was used as a control to normalize HUWE1 mRNA levels in different cell groups. HUWE1 mRNA levels of F6 and G10 were decreased by $\sim 75\%$ compared to the vector control group, which was consistently shown by all three HUWE1 primer sets (Figure 3.4.A). These results suggested that HUWE1 mRNA was successfully knocked down in both F6 and G10 cells.

Next, I examined the impact of HUWE1 KD on PARP-1 mRNA level. Two different PARP-1 primers targeting the N-terminal, and C-terminal regions of PARP-1 cDNA were used to determine the mRNA levels of PARP-1 and GAPDH was used as a control to normalize PARP-1 mRNA levels in different cell groups. As shown in Figure 3.4.B, there were no statistically significant variation in mRNA level in VE, F6 and G10 transduced U2OS cells. This result suggested that HUWE1 knockdown did not have effect on PARP-1 mRNA level.

Previously, I have shown that ectopically expressed HUWE1 was the E3 ligase for PARP-1. HUWE1 could ubiquitinate PARP-1, leading to PARP-1 degradation via 26S proteasome. Therefore, I hypothesized that the HUWE KD would stabilize PARP-1 in U2OS. To test this hypothesis, VE, F6 and G10 cells were subject to immunoblotting to detect the steady-state protein levels of HUWE1 and PARP-1. HUWE1 protein was not visible from F6 and G10 groups compared to VE, which indicated a successful knock down of HUWE1 from the protein level (Figure 3.4.C). However, the steady-state levels of PARP-1 in F6 and G10 were elevated compared to the VE group. Figure 3.4.C showed that the PARP-1 protein levels indeed increased significantly in the HUWE1 KD groups indicated by three individual experiments. Taken together, the HUWE1 KD could stabilize the PARP-1 protein without affecting the PARP-1 mRNA level.

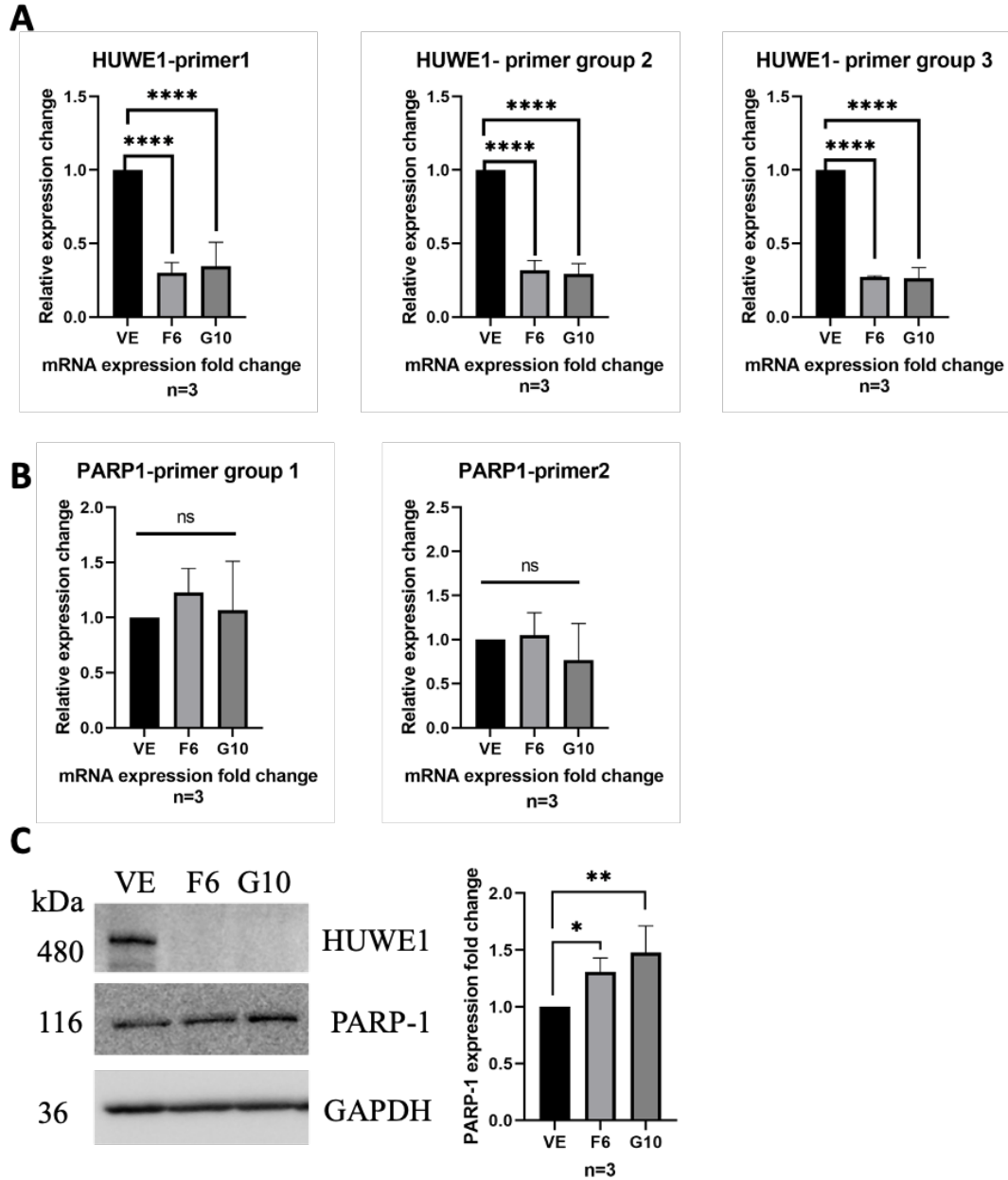
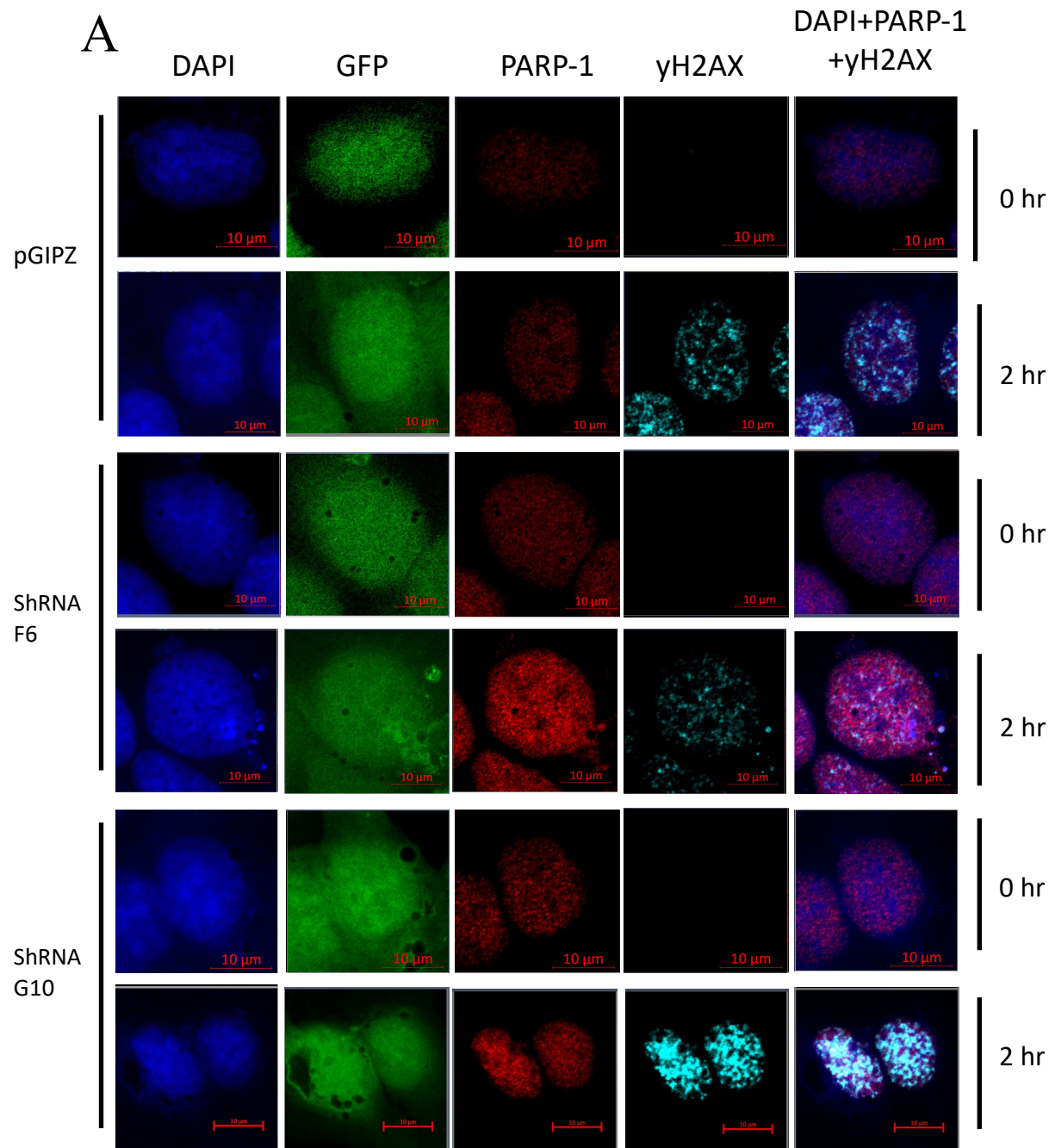


Figure 3.4 HUWE1 knockdown stabilized PARP-1 in protein level. U2OS cells were infected with either empty vector (VE), HUWE1 ShRNA F6 or G10. Three groups of HUWE1 primers (A) and two groups of PARP-1 primers (B) were used. U2OS cell mRNA was isolated and subjected to qRT-PCR. C, Total cell lysate of U2OS cells with either empty vector (VE), HUWE1 ShRNA F6 or G10 were subject to immunoblotting for HUWE1 and PARP-1. The statistical analysis was using One way ANOVA. The calculated p values were indicated as, ns, no significant differences, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.5 HUWE1 knockdown increased the nuclear PARP-1 level in U2OS cells.

To further investigate the role of HUWE1 in the regulation of PARP-1 in response to DNA damage, confocal imaging was used to study the effect of HUWE1 knockdown on the cellular PARP-1 signals with or without etoposide induced DNA damage (Figure 3.5). The γ H2AX staining was used as the DNA damage marker following etoposide treatment. In the absence of etoposide treatment, the PARP-1 signal appeared to nuclear and quite low in the VE cells, and the PARP-1 signal significantly increased in HUWE1 KO F6 and G10 cells. Following 2-hour etoposide treatment, the PARP-1 level from VE group did not show significant change compared to the untreated VE group, despite being more distinct and confined. In contrast, a significant increase of PARP-1 signal was observed in both F6 and G10 groups, which were further intensified following etoposide treatment (Figure 3.5). This result indicated that PARP-1 became more stable in HUWE1 KD groups and accumulated in the nucleus following DNA damage.



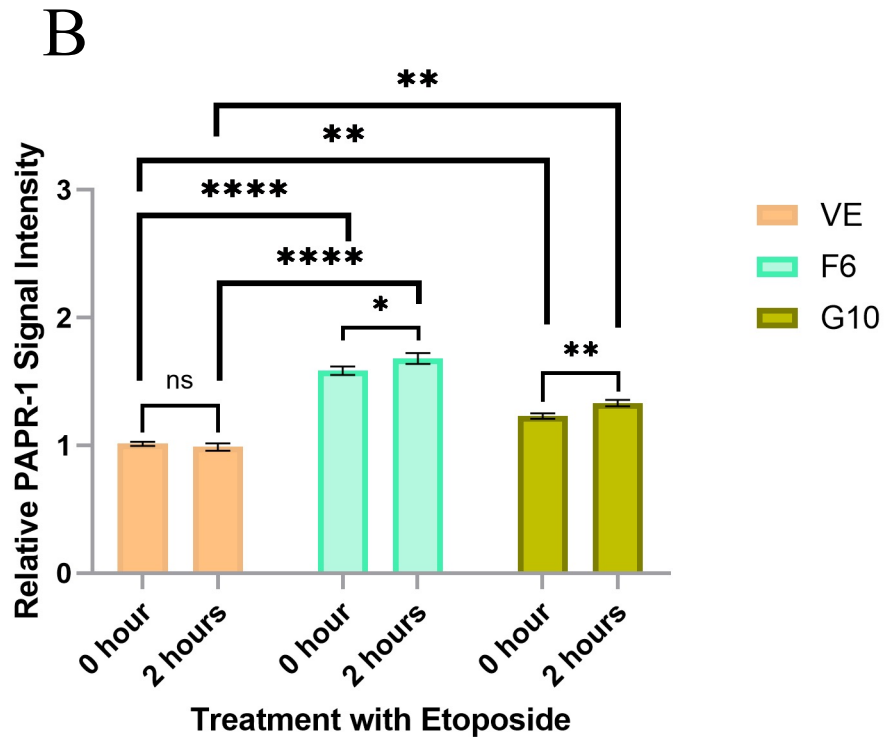


Figure 3.5 HUWE1 knockdown stabilized PARP-1 protein.

U2OS cells were transfected with either empty vector (VE) or HUWE1 ShRNA F6 or HUWE1 ShRNA G10. A, U2OS cells were subjected to IF to detect the PARP-1 and γ H2AX protein levels. B, statistics for A. U2OS cells either treated with 10 μ M etoposide for 2 hours to induce DNA lesion or with DMSO treatment and visualized by confocal, 100-200 cells were counted for each group, the 0hr VE group was normalized to one. (Scale bar 10 μ m).

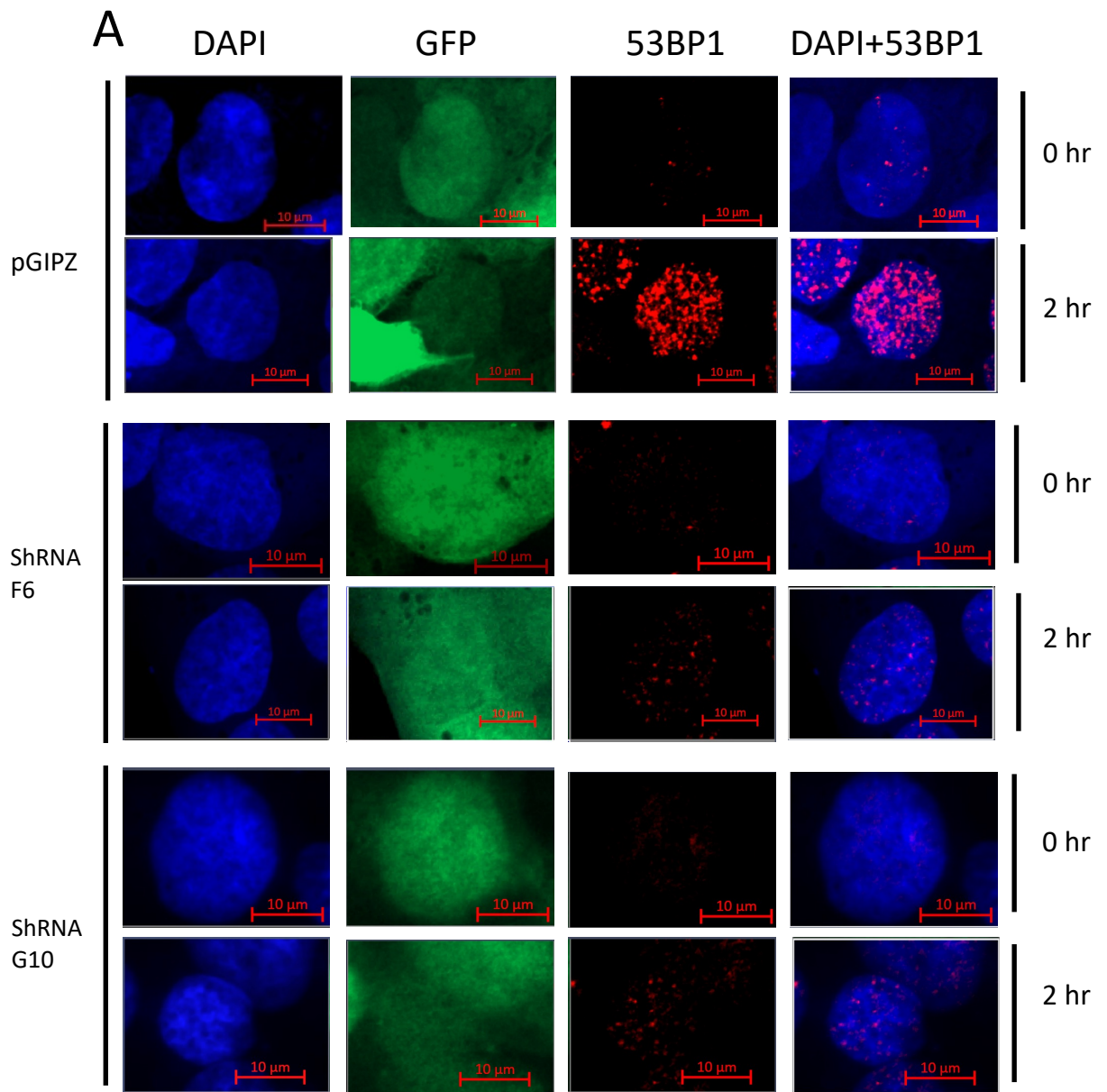
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Multi-comparison test was performed to calculate p values.

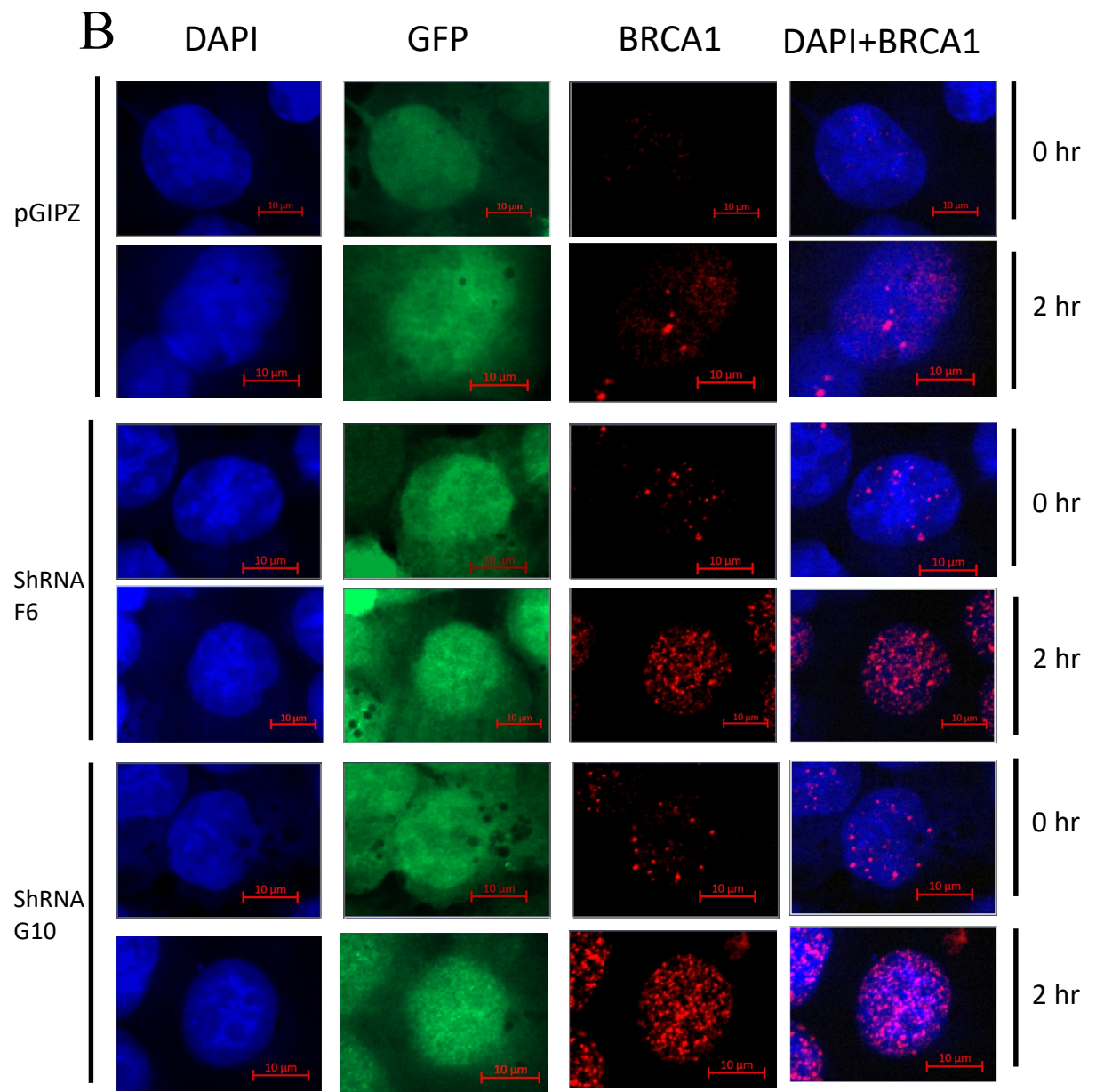
3.6 HUWE1 knockdown resulted in increased BRCA1 and decreased 53BP1 foci in the nuclei following DNA damage.

HUWE1 has been reported as a DDR regulatory protein involved in the DDR signaling pathway. BRCA1 and 53BP1 are two of the most critical proteins involved in repairing DNA double strand breakage. In DSB, many DNA foci will be formed on the DNA lesions by accumulation of various DDR proteins such as 53BP1 (Aprigliano et al., 2021). HUWE1 has been shown to ubiquitinate BRCA1 *in vitro* (Jang et al., 2021). However, the association between HUWE1 and nuclear 53BP1 and BRCA1 has not been studied before.

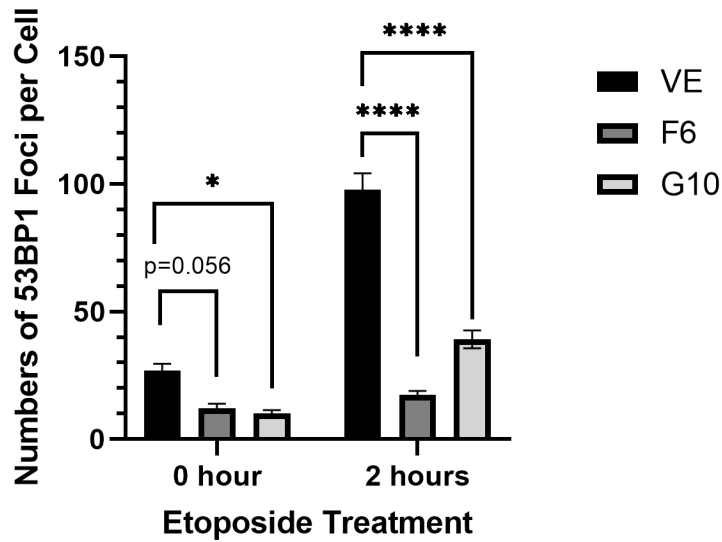
I used confocal microscopy to study 53BP1 associated DNA foci formation and the signal intensity of BRCA1 following induction of DNA damage in HUWE1 KD cells. U2OS cells expressing GFP-VE, HUWE1 shRNA F6 and G10 cells were processed for IF imaging as described in material and method 2.1, using the 53BP1 and BRCA1 specific antibody.

To quantify the 53BP1 foci formation, 100-150 cells were counted from each group and the statistical analysis results were shown in Figure 3.6.A and C. HUWE1 KD significantly decreased the 53BP1 foci formation, suggesting HUWE1 KD impaired DDR signaling. By contrast, A significant increase of BRCA1 (Figure 3.6.B and D) was observed for F6 and G10 groups with or without etoposide treatment, which was consistent with the role of HUWE1 as an E3 ligase regulating BRCA1 stability.





C



D

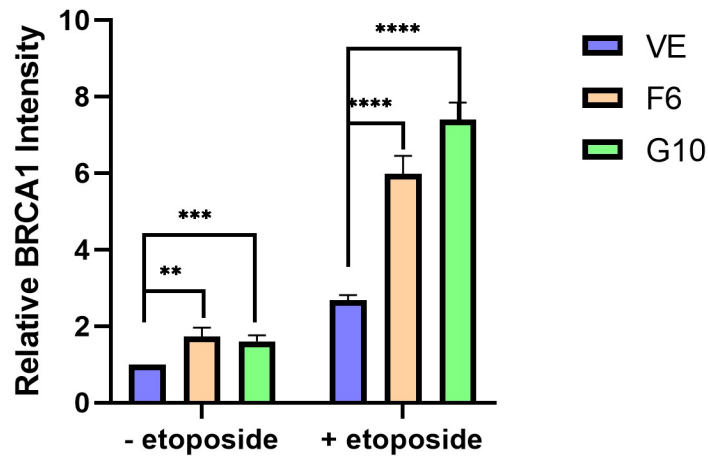


Figure 3.6 HUWE1 knockdown resulted increased BRCA1 and decreased 53BP1 protein stabilities. U2OS cells were transfected with either empty vector (VE) or HUWE1 ShRNA F6 or HUWE1 ShRNA G10. U2OS cells either treated with 10 μ M etoposide for 2 hours to induce DNA lesion or with DMSO treatment and visualized by confocal, 100-150 cells were counted for each group, the 0hr VE group was normalized to one. A and B, Confocal imaging to visualize the BRCA1 and 53BP1 protein levels. C and D, statistics for the 53BP1 foci formation from A and the relative BRCA1 signal changes from B. (scale bar 10 μ m).

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Multi-comparison test was performed to calculate p values.

3.7 HUWE1 and PARP-1 showed a reversed protein expression pattern following induction of DNA damage by etoposide.

HUWE1 has been shown as a PARP-1 E3 ligase, to ubiquitinate PARP-1 in the cell. Therefore, I hypothesized during DNA damage signaling, HUWE1 may play a role to remove PARP-1 from DNA lesions and inhibit PARP-1 overactivation.

To test this hypothesis, wild type U2OS cells were treated with 10 μ M etoposide for different time points from 15 minutes to 2 hours. Then the cells were harvested and lysed by RIPA buffer for total cell lysate, or fractionation to differ cytosolic and nuclear proteins. The samples were then subjected to immunoblotting to visualize the HUWE1 and PARP-1 protein levels from whole cell lysate, cytosolic, and nuclear fractions.

Upon induction of DNA damage by etoposide, I observed that the HUWE1 level increased at 30-min and 1-h time points in total cell lysate and decreased after 2 hours. This change of HUWE1 was not obvious in the cytoplasmic fraction. Interestingly, in the nuclear fraction, the level of HUWE1 gradually increased following induction of DNA damage. Unlike HUWE1, which was present in both cytoplasm and nucleus, PARP-1 was only found in the nucleus. The PARP-1 level was not changed in the total cell lysate after induction of DNA damage. However, the nuclear fraction of PARP-1 revealed a mild increase at 30 mins and a gradual decrease at 1 and 2 hour time points (Figure 3.7.B)

To further explore the relationship between HUWE1 and PARP-1 in the course of DNA repair, I induced DNA damage with 10 μ M etoposide in U2OS cells and monitored the levels of HUWE1 and PARP-1 from 15 minutes to 24 hours. A clear reversed HUWE1 and PARP-1 protein pattern was observed within the nuclear fraction. PARP-1 protein level was relatively high before induction of DNA damage, shortly increased after etoposide treatment, then slowly

declined after four hours, and finally returned to time zero after 24 hours. In contrast, HUWE1 protein level was low at time zero, gradually increased following etoposide treatment, and it's peaked at 2-h and 4-h time points coincided with the decline of PARP-1. This result strongly suggested that the role of HUWE1 in the regulation of PARP-1 and preventing PARP-1 accumulation and overactivation in the nucleus following DNA damage (Figure 3.7.A).

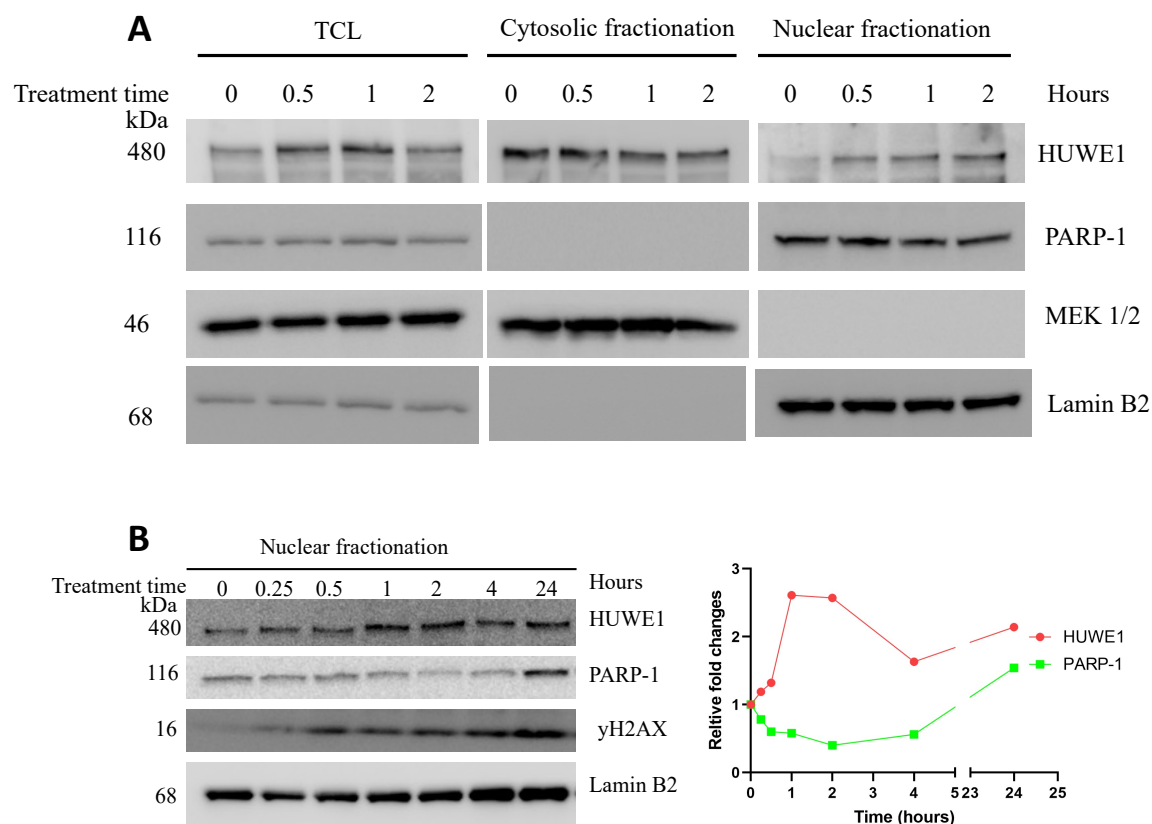


Figure 3.7 HUWE1 and PARP-1 expression levels in response to DDR.

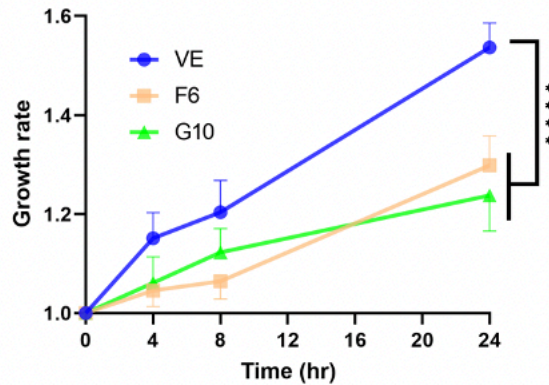
U2OS cells treated with 10 μ M etoposide for different time points. A, 10% of the total cells were lysed by RIPA buffer for total cell lysate immunoblot (TCL). The rest cells were processed by Fractionation Extraction Kit to collect cytosolic proteins. The nuclear pellets were lysed directly by RIPA buffer to isolate nuclear proteins. B, the time dependent nuclear fractionation for HUWE1 and PARP-1 and the signal quantification for PARP-1 and HUWE1. At 0 time points, the PARP-1 and HUWE1 signals were normalized to 1.

3.8 The effect of HUWE1 KD on DNA damage induced cell death in the U2OS cell

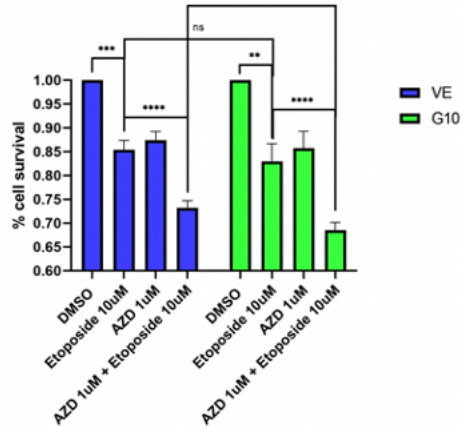
As HUWE1 KD stabilized PARP-1 and BRCA1, but decreased 53BP1 foci following induction of DNA damage, I hypothesized that the HUWE1 KD will sensitize the U2OS cells to the DNA damage. In this experiment, cell titer Glo 2.0 is a chemical reagent to interact with ATP which indicates the mitochondrial metabolic activity in the cells. The illuminances reading is proportional to the cell viability. Without drug treatment, the shHUWE1 F6 and G10 cells showed significant slower growth compared to the vector control group. When the cells were treated with etoposide, F6 and G10 groups showed a mild but significant sensitization to etoposide induced DNA damage compared to VE group.

AZD is a clinically approved potent PARP inhibitor, used to treat HR deficient breast and ovarian cancers. It inhibits PARP-1 activity and traps PARP-1 on DNA lesions to block DNA repair. To investigate the effect of HUWE1 knockdown on AZD drug sensitivity in U2OS cells, we treated cells with 1 μ M AZD. The vector and the shHUWE1 group showed similar sensitivity to AZD treatment. Cell survival decreased to approximately 85% following 24 h AZD treatment. When the cells were treated with both etoposide (10 μ M) and AZD (1 μ M), shHUWE1 cells were significantly more sensitive to the double treatment. It suggests that HUWE1 knockdown sensitize cells to the DNA damage, which could work synergistically with PARP-1 inhibitor to block DNA damage repair.

A



B



C

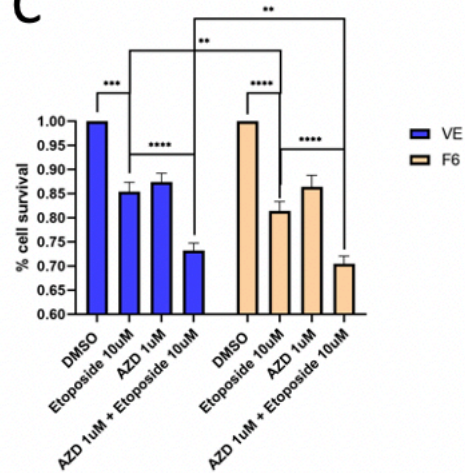


Figure 3.8, HUWE1 KD sensitize the U2OS cell survival rate. A, F6, G10 and VE cells were seeded into 96 well plate and the cell titer glo 2.0 were used to measure the cell growth rate. B and C, the cells were treated with DMSO only, etoposide 10μM, AZD 1μM or drug combination for 24 hours. The luminescence signals were obtained by cell titer glo 2.0 kit and obtained the bioluminescence signals at 528/20 wavelength. The DMSO treated F6, G10 and VE groups were normalized to 1. Three quantitative repeats were performed for this Figure. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Multi-comparison test was performed to calculate p values.

Chapter 4 : Discussion and future directions

PARP-1 is an important protein involved in DDR signaling pathway. PARP-1 is extremely sensitive in detecting DNA lesion. Upon DNA damage, it will be recruited to the damaged sites, where it is activated and begins to synthesis PAR chain. The PAR chains will be added to modify PARP-1 and other DNA damage signaling proteins. However, overactivation of PARP-1 leads to excessive synthesis of PAR polymers and the PAR will transmigrate from nucleus into cytosol. The cytosolic PAR, along with NAD⁺ and ATP consumption, can trigger mitochondria to release apoptosis inducing factor and result in irreversible cell death which is called Parthanatos. Therefore, ubiquitination mediated destabilization and degradation of PARP-1 by proteasome is an important mechanism to prevent PARP-1 overactivation and maintain cellular homeostasis. In the ubiquitination pathway, E3 ligases play a very critical role in specific protein ubiquitination and subsequent degradation by the proteasome. Dysregulation of PARP-1 associated E3 ligase will impair normal functions of PARP-1. Therefore, identifying the PARP-1 associated E3 ligases and investigating their interactions is critical for elucidating cellular signaling pathways in response to DNA damage. HUWE1 is an E3 ligase that has been reported to be involved in the DNA damage repair pathway. However, the exact roles of HUWE1 in DDR pathway are not understood. Therefore, the purpose of this research is to characterize the interaction between these two proteins and their roles in the DDR pathway.

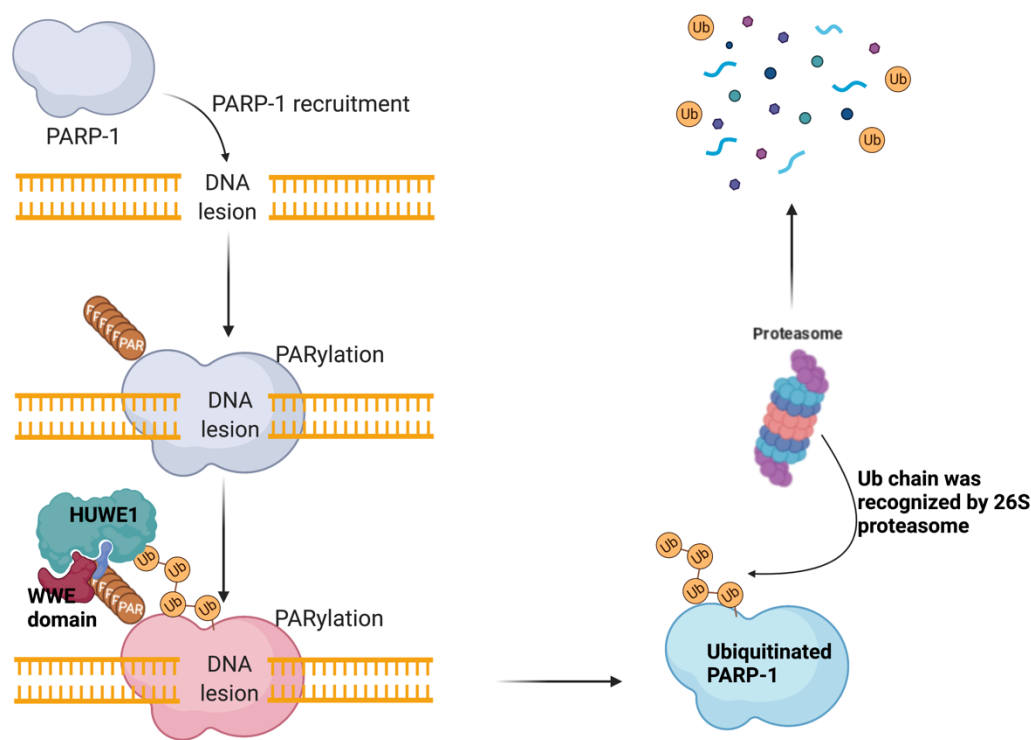


Figure 4.1 schematic summary of the research. The PARP-1 was recruited to the DNA lesion and synthesis PAR chains. The HUWE1 was attracted and bind to PARP-1 with the assistance of PAR, subsequently the PARP-1 was ubiquitinated by HUWE1. The Poly-ubiquitinated PARP-1 can be recognized by 26S proteasome and degraded.

4.1 HUWE1 is an E3 ligase for PARP-1

HUWE1 shared some critical features with Iduna, another PARP-1 associated E3 ligase. Both contain a PAR reader motif WWE1 domain. Therefore, the HUWE1 was hypothesized to be a PARP-1 E3 ligase protein by direct binding to the PAR chain. I overexpressed FLAG-HUWE1 WT and its WWE domain mutants in order to perform immunoprecipitation assays to explore

whether HUWE1 interacts with PARP-1 and whether WWE1 is indispensable for the interaction. The reciprocal immunoprecipitation experiments using FLAG and PAR antibodies revealed an interaction between HUWE1 and PAR following etoposide induced DNA damage. I further showed that the HUWE1 can interact with activated PARP-1 via its WWE1 domain.

Next, I hypothesized that the interaction between HUWE1 and PARP-1 could lead to the HUWE1 associated ubiquitination of PARP-1, which would lead to 26S proteasome mediated PARP-1 degradation. Therefore, to investigate the PARP-1 ubiquitination, etoposide and the proteasomal inhibitor MG132 treatment were applied to 293T cells. When the 26S proteasome is inhibited, the polyubiquitinated PARP-1 can be detected by PARP-1 immunoprecipitation and visualized by immunoblotting with a ubiquitin antibody. Several lines of evidence from this study support the role of HUWE1 as PARP-1 E3 ligase to regulate its stability. (1) Figure 3.2.B showed an extremely strong ubiquitinated PARP-1 in FLAG-HUWE1 WT group compared to other mutant or control groups. It suggested that PARP-1 became more ubiquitinated when wild type HUWE1 was overexpressed. (2) HUWE1 mediated PARP-1 ubiquitination is dependent on the catalytic activity of HUWE1 and its WWE domain, as the catalytically negative mutant and WWE mutants RA and YA were not able to ubiquitinate PARP-1. Additionally, from the total cell lysate (Figure 3.1.A, Figure 3.2.A and Figure 3.3.A), the overall PARP-1 expression level decreased in WT FLAG-HUWE1 compared to 293T cells and two RA and YA HUWE1 WWE domain mutants. This suggested that the overexpression of WT HUWE1 can destabilize PARP-1 protein level. Therefore, HUWE1 is a specific PARP-1 E3 ligase that can ubiquitinate PARP-1 and destabilize PARP-1.

In addition, the integrity of HUWE1 WWE domain was shown to be critical in modulating the binding between HUWE1 and PARP-1/PAR and ubiquitination of PARP-1. It

suggested that HUWE1 may only act on the activated PARP-1 and prevent PARP-1 overactivation rather than destabilizing unmodified PARP-1 proteins. When PARP-1 was activated, PAR chains were synthesized and at the same time, the PAR chain functioned as a signal to recruit HUWE1 to prevent overactivation of PARP-1. When PARP-1 was not active, only very limited PAR chains were presented within the cells, and thus HUWE1 was not needed for PARP-1 ubiquitination.

4.2 HUWE1 KD can increase PARP-1 protein stability

HUWE1 was proven to be a specific PARP-1 E3 ligase, therefore HUWE1 knockdown would increase PARP-1 stability. Two shRNAs were used to decrease cellular HUWE1 protein level. Indeed, a significant PARP-1 protein increase was observed in the shRNA groups compared to the empty vector group (Figure 3.5). More interestingly, after two hours of etoposide treatment, the empty vector group did not show a significant difference in PARP-1 protein level fold change (Figure 3.5). By contrast, both F6 and G10 groups showed a significant increase of PARP-1 level after etoposide treatment (Figure 3.5). This implied the PARP-1 protein level increased during the etoposide induced DNA lesion, and the HUWE1 protein acts to inhibit PARP-1 from overactivation. Also, the qRT-PCR data (Figure 3.4.A) showed that the PARP-1 mRNA level was not compromised by HUWE1 KD, therefore the HUWE1 KD can impact PARP-1 protein stability post-translationally without any interference on PARP-1 transcription.

4.3 HUWE1 was involved in both HR and NHEJ repair pathway

BRCA1 and 53BP1 are two major proteins involved in HR and NHEJ. BRCA1 is the key protein involved in HR and it can promote the clearance of 53BP1 in S phase. 53BP1 is a key protein determining whether the DNA damaged cells undergo HR or NHEJ. Considering HUWE1 was intensively involved in DDR, it was therefore necessary to check whether HUWE1

could impact these two critical DDR associated proteins. To test the hypothesis, IF was applied to HUWE1 shRNA and empty vector groups to observe the foci formation of 53BP1 and the protein level differences of BRCA1 in the presence or absence of etoposide to induce DNA lesions.

The KD of HUWE1 could impact both two critical proteins (Figure 3.6). The HUWE1 KD could decrease 53BP1 foci formation (Figure 3.6.A and C), meanwhile, it could increase the stability of BRCA1 (Figure 3.6.B and D). This result indicated that HUWE1 KD could participate in multiple steps of DNA lesion repair from NHEJ to HR.

The Cell Titer showed that the HUWE1 KD would sensitize the cells from DBS which was induced by etoposide. It seemed that the HUWE1 KD could, on one hand to signal the DNA lesion repair to HR pathway as indicated by increased BRCA1 signal following etoposide treatment, on the other hand to sensitize the cells targeting PARP-1 mediated DDR pathway. Therefore, it is important for us to further explore a full scope of the role of HUWE1 in DDR.

4.4 HUWE1 negatively regulated PARP-1 level during DNA lesion

As can be seen from the cellular fractionation assay, without DNA damage, the HUWE1 predominantly resided in cytosol. Though the cultured cells experienced simultaneously endogenous DNA lesions, this mild to moderate DNA stress was not strong enough to initiate HUWE1 migration, therefore the HUWE1 signal from the nucleus is almost invisible. However, by the treatment of chemotherapeutic drug etoposide to induce massive DNA damage, the cytosolic HUWE1 protein signal dropped gradually whereas the nuclear HUWE1 signal became visible and stronger following the time of treatment. This result suggested that, upon DNA damage, the HUWE1 migrates from cytosol into nucleus.

Though the HUWE1 migrated from the cytosol into nucleus, the overall expression of HUWE1 protein during DNA lesion was more interesting. The total cell lysate immunoblots revealed an opposite pattern for HUWE1 and PARP-1 protein following induction of DNA damage. For short time of etoposide treatment (0-2 hours), the overall HUWE1 protein expression increased, whereas the PARP-1 protein showed a brief mild increase then gradually dropped. This can be explained that the HUWE1 sensed the activation of PARP-1 and functioned to ubiquitinate PARP-1, the PARP-1 subsequently degraded by 26S proteasome. However, over longer time etoposide treatment, I observed that the overall protein level of HUWE1 and PARP-1 were restored.

4.5 Future directions

By completion of my research, a great many questions arose waiting for further exploration. The HUWE1 can negatively regulate both BRCA1 and PARP-1, which are critical proteins for HR and NHEJ during DNA DSB. Additionally, the data have shown that HUWE1 KD increased BRCA1 and PARP-1 protein level at the same time. At the meantime, HUWE1 KD can decrease 53BP1 protein level which promote the DSB goes to HR repairing which is beneficial to the cells under DSB. Therefore, the HUWE1 KD should promote at least the HR, and partly NHEJ (the NHEJ level decreased characterized by decreased 53BP1 but the efficiency increased characterized by increased PARP-1) under DNA lesion conditions induced by etoposide treatment. However, the HUWE1 KD in U2OS cells sensitized these cells to etoposide treatment which could induce DBS detected by Cell Titer Glo experiment. There must be some critical mechanisms waiting us to reveal.

For the future experiments to understand the role of HUWE1 in DDR, we can knockdown both HUWE1 and either PARP-1 or BRCA1. By double protein KD, we can explore how HUWE1 impacts cell viability in PARP-1 and BRCA1 mediated DDR pathway in more depth.

At the same time, mitochondria are another important organelle for cell death, many critical proteins, such as apoptosis inducing factor, caspase family proteins, are all related to mitochondria. Some researchers have been focusing on the role of HUWE1 in mitochondrial functions, and some indirect evidence have pointed towards mitochondria as well. Parthanatos is a newly discovered caspase independent programmed cell death which is associated with overactivation of PARP-1. Therefore, it definitely worth exploring the connection between HUWE1 and mitochondria, particularly under DNA damage.

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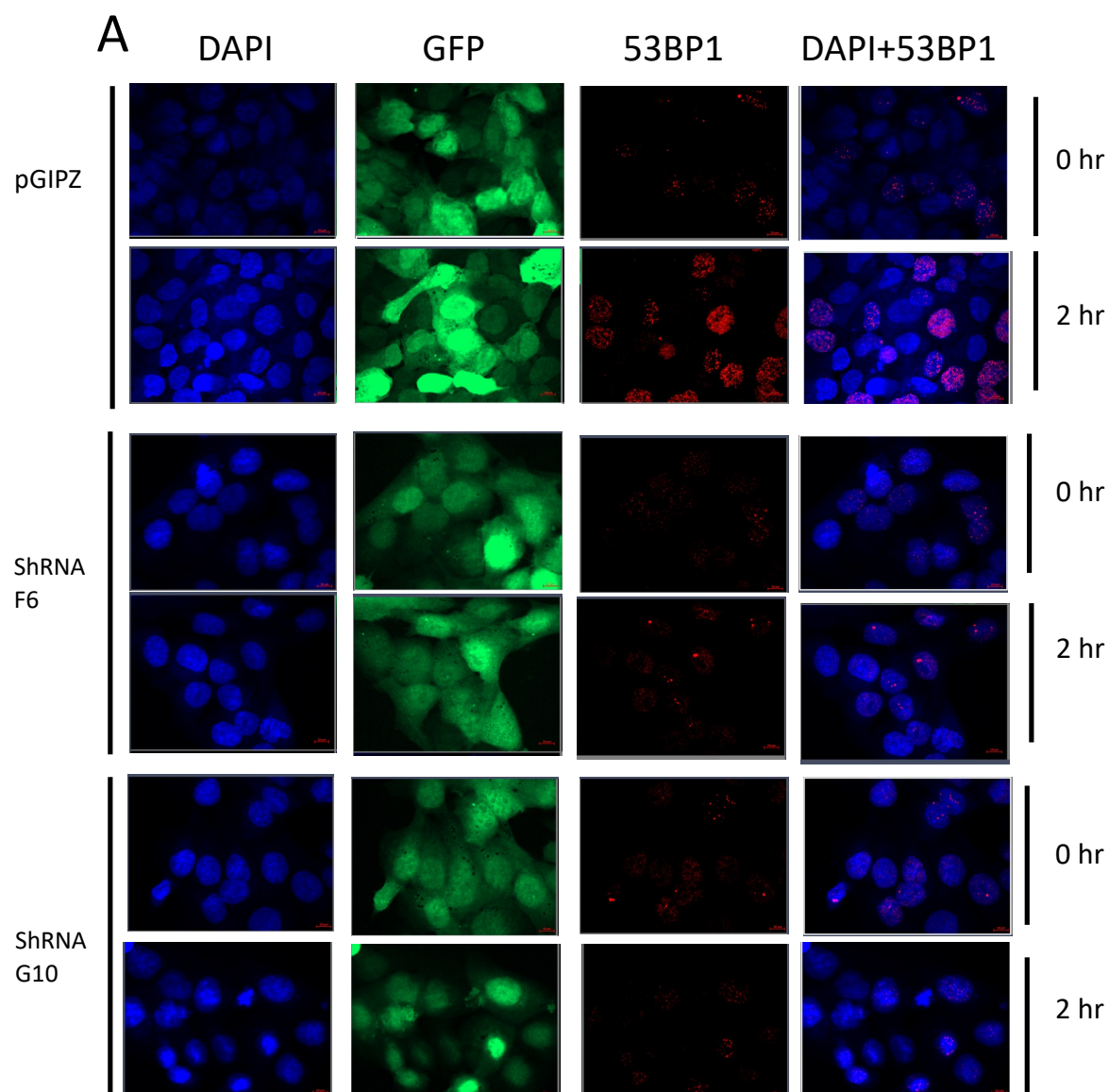
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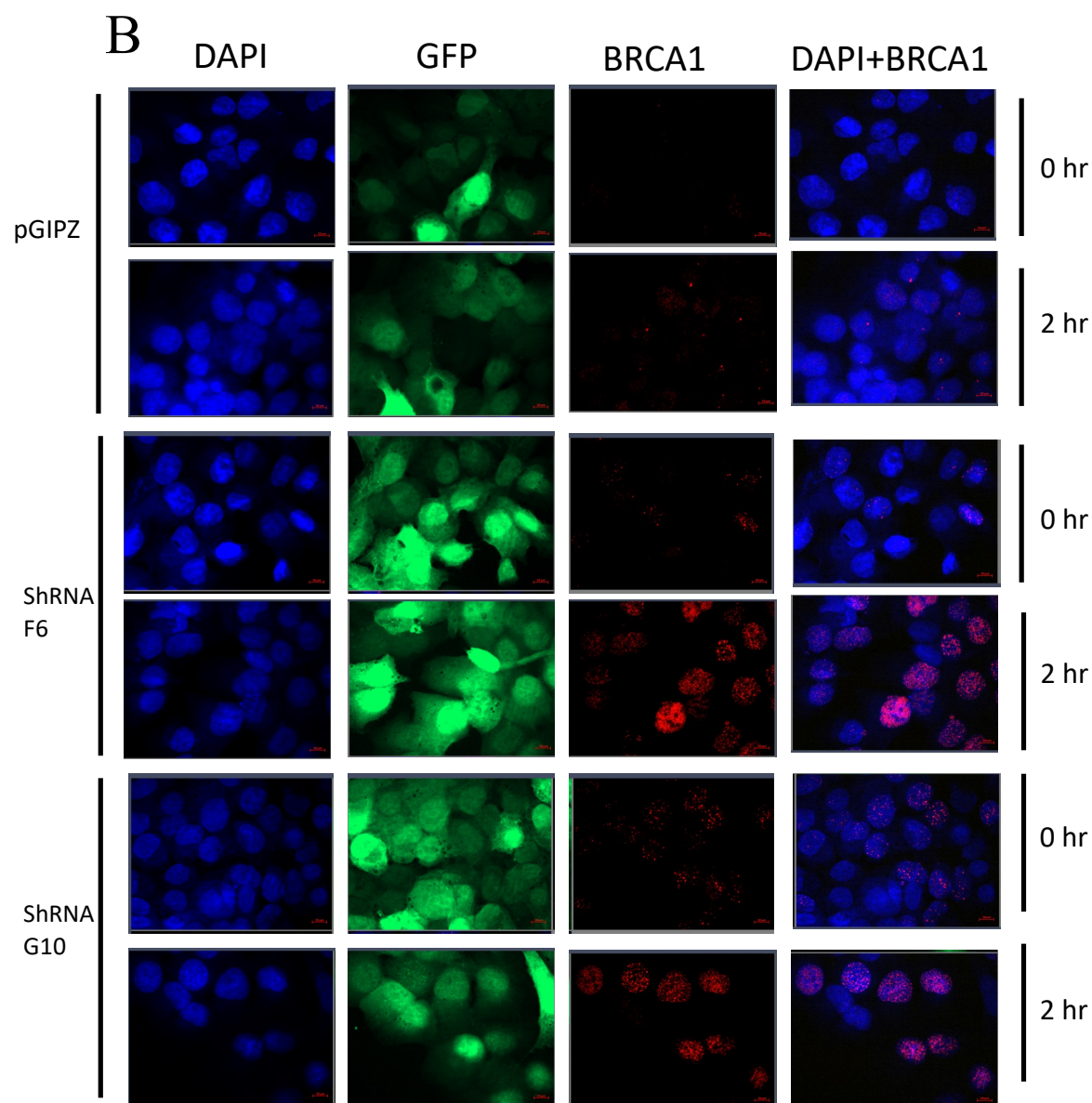
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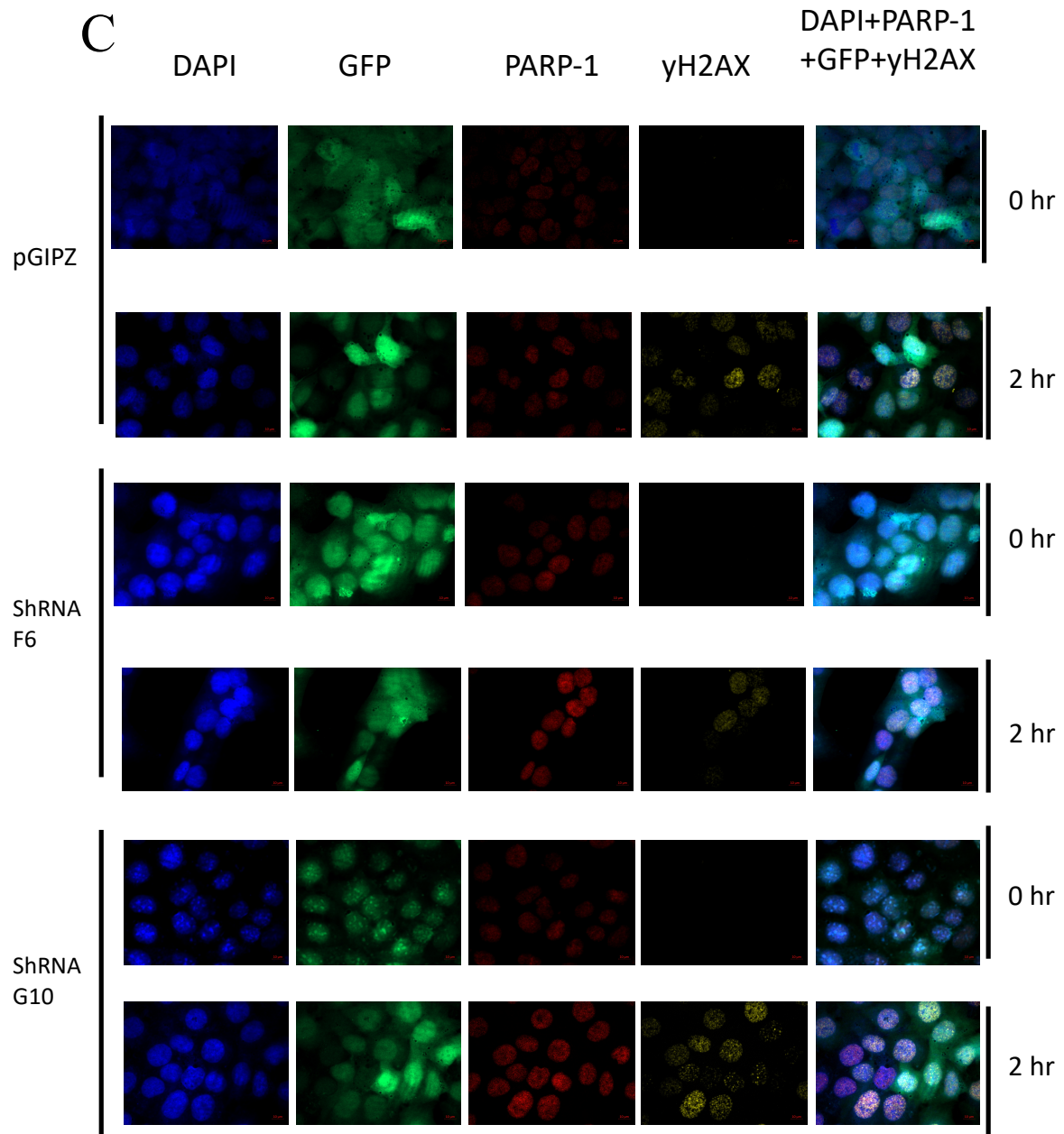
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Appendix

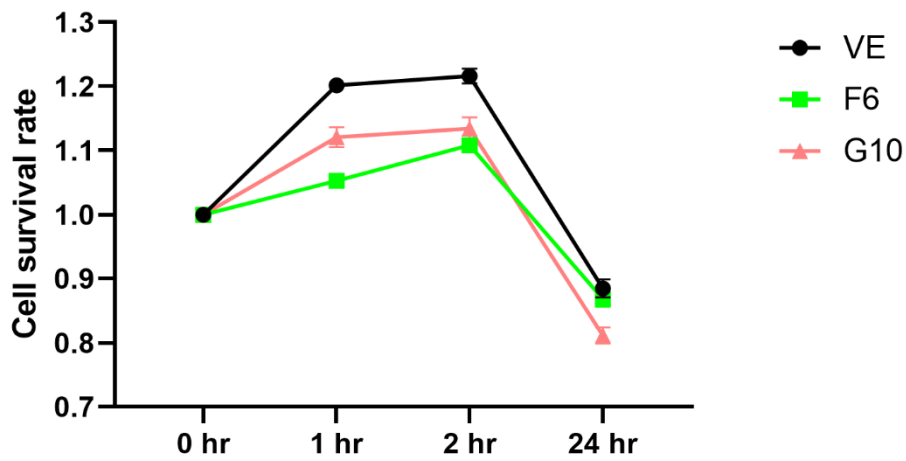
Supplementary data







Suppl 1, The full frame IF images supplemented for Figure 3.5 and Figure 3.6. U2OS cells were transfected with either empty vector (VE) or HUWE1 ShRNA F6 or HUWE1 ShRNA G10. U2OS cells either treated with 10 μ M etoposide for 2 hours to induce DNA lesion or without etoposide treatment and visualized by confocal. A, 53BP1 staining. B, BRCA1 staining. C, PARP-1 and γ H2AX staining.



Suppl 2, The time course dependent cell growth rate. U2OS cells were transfected with either empty vector (VE) or HUWE1 ShRNA F6 or HUWE1 ShRNA G10. The cells were treated with 1uM AZD for different time course and the cell populations were measured by Cell Titer Glo2.0.