INVESTIGATING THE ROLE OF THE E3 LIGASE HUWE1 IN RESPONSE TO DNA DAMAGE

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Abstract

HUWE1 (HECT, UBA, WWE domain containing 1) is a HECT-domain E3 ligase that is involved in ubiquitin mediated degradation and signaling in a variety of cellular processes including apoptosis, DNA replication, and recently shown to be involved in DNA damage repair. However, the role of HUWE1 in the DNA damage response (DDR) pathway has not been well understood. PARP1 (poly-ADP ribose polymerase 1), is a key protein involved in sensing and initiating DNA damage signaling through catalyzing the attachment of poly-ADP ribose polymers (PAR chains) to its substrate proteins. PARP1 has been shown to bind DNA and mediate the recruitment of many DDR proteins to facilitate repair at the DNA damage site. HUWE1 was recently found to bind ADP-ribose (single unit of PAR chains) through its WWE domain. Since PARP1 plays a large role in the DDR, it was hypothesized that HUWE1 and PARP1 could be functionally associated in the regulation of protein signaling in the DDR pathway. To better understand the role of HUWE1 in the regulation of DDR, the association of HUWE1 with DDR proteins including PARP1 was studied using biochemical and cellular approaches. In this study, HUWE1 was shown to be involved in the initiation of DNA damage signaling through affecting phosphorylated-ATM and phosphorylated-NBS1 in response to DNA damage. Moreover, HUWE1 was shown to interact with PAR and PARP1 using co-immunoprecipitations. The association of HUWE1 and PARP1 led to PARP1 destabilization and degradation in U2OS cells. Collectively, this study revealed that HUWE1 is a novel E3 ligase of PARP1, which provides a new mechanistic insight into the signaling network of cellular DNA damage response. As PARP1 inhibitors have been approved for the treatment of DNA repair deficient cancers, understanding the regulation of PARP1 and DNA repair by HUWE1, could be used to improve therapies targeting the DDR pathways.

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I would like to dedicate this thesis in the loving memory of my grandfather. Thank you for believing in me, encouraging me to always follow my passion, and being proud of my accomplishments big or small!

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Chapter 1: Introduction

Genome stability is important for the maintenance and replication of healthy cells (Hoeijmakers 2009). Cells are constantly exposed to both endogenous and exogenous stress. These stresses can promote DNA damage and the accumulation of mutations, leading to genomic instability, which could result in carcinogenesis or cell death (Hoeijmakers 2009). In order to manage DNA damage in cells and the response to exogenous or endogenous stressors, the cell has adapted an intricate DNA Damage Response (DDR) in order to mediate DNA repair (Friedberg, McDaniel, and Schultz 2004; Lindahl 1993). The DDR is comprised of protein sensors, mediators, and effectors that recognize damaged DNA, initiate cellular signaling and facilitate DNA repair (S. P. Jackson and Bartek 2009). These pathways are highly intricate and are dependent on the type of DNA damage incurred. Mutations in the DDR pathways and associated genes can result in aberrant DNA damage sensing and repair, which can ultimately promote carcinogenesis (Hoeijmakers 2009; Tubbs and Nussenzweig 2017).

1.1. Types of Cellular DNA Damage

The types of DNA damage caused by endogenous and exogenous stress vary greatly in cells. Endogenous sources of DNA damage include incorrect DNA base insertion during replication, DNA base deamination (ex. cytosine deamination; Cytosine→Uracil base switch), and DNA depurination or depyrimidination (missing nucleotide base) (Ciccia and Elledge 2010; A. L. Jackson and Loeb 2001; Nakamura and Swenberg 1999). Exogenous sources of DNA damage can occur though both chemical exposure and environmental conditions (Ciccia and Elledge 2010). Examples of exogenous sources of DNA damage include ultraviolet radiation (UV), ionizing radiation (IR) and exposure to chemical agents including chemotherapy drugs (Ciccia and Elledge 2010; Mehta and Haber 2014). Exposure to such agents can cause genotoxic stress

to cells resulting in various types of DNA damage including pyrimidine dimers, single stranded breaks (SSBs) and double stranded breaks (DSBs) (Ciccia and Elledge 2010). Exposure to UV radiation causes pyrimidine dimers, and accumulated secondary SSBs, and DSBs (Halicka et al. 2005; Rastogi et al. 2010). IR produces oxidative radicals that nick the DNA backbone resulting in SSBs, and high doses of IR could produce multiple adjacent nicks in the DNA leading to DSBs (Ciccia and Elledge 2010; Mehta and Haber 2014). Moreover, chemotherapy drugs can cause SSBs and DSBs through various mechanisms. These lesions can occur as a result of alkylating agents (ex. Methyl methhanesulfonate), inter-strand and intra-strand crosslinking (ex. Cisplatin), topoisomerase inhibitors (ex. Etoposide), and DNA polymerase and deoxyribonuclease inhibition (ex. Hydroxyurea) (Alakananda Basu and Krishnamurthy 2010; Ciccia and Elledge 2010; Mehta and Haber 2014; Olive and Banáth 2009; Soubeyrand, Pope, and Haché 2010). Lastly, the oxidation of DNA by reactive oxygen species (ROS) primarily leads to Guanine→Thymine base mutations and SSBs, which can occur through both endogenous or exogenous sources previously mentioned (Calderón-Garcidueñas et al. 1999; Cheng et al. 1992; Spencer et al. 1996; Tubbs and Nussenzweig 2017).

1.2. Cellular DNA Damage Repair Pathways

To cope with different types of DNA lesions, cells have evolved multiple mechanisms and pathways to facilitate DNA repair. Mismatch repair (MMR), Base excision repair (BER) and Nucleotide excision repair (NER) are examples of DDR pathways used to repair small and confined DNA structural lesions (Ciccia and Elledge 2010). MMR fixes mis-paired nucleotide bases, BER recognizes and repairs chemically altered nucleotides, and NER facilitates the excision of small oligonucleotides (up to ~30 base pairs) in DNA lesions (Ciccia and Elledge

2010; Hoeijmakers 2009). SSBs and DSBs are common types of DNA lesions produced by exogenous DNA damage agents and are harmful to the genomic stability of the cell. SSB repair is facilitated by the single stranded break repair (SSBR) pathway, which involves many enzymes and signaling proteins that overlap with BER and therefore is known as BER/SSBR. DSBs are repaired through homologous recombination (HR) or non-homologous end joining (NHEJ) (Keith W Caldecott 2008; Mehta and Haber 2014). Therefore, MMR, NER, BER, SSBR, HR and NHEJ are the main pathways that the cell utilizes to facilitate appropriate DNA repair (Figure 1.1). Each pathway mediates a unique signaling response to different types of DNA damage. Coordination between these various pathways is important in order to mediate DNA repair and maintain genomic integrity. Thus, various common pathways will be discussed in further detail below.

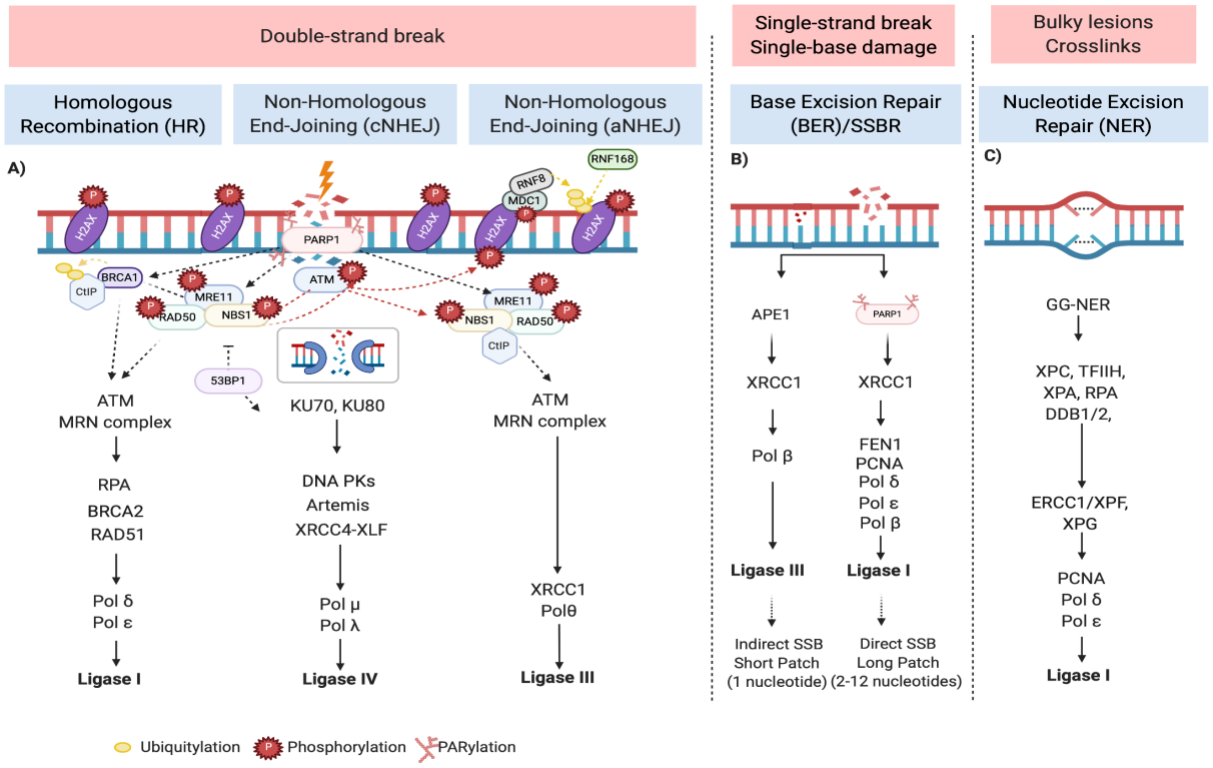


Figure 1.1. DNA repair pathways for various types of DNA damage. Overview of key proteins involved in A) DSB Repair (HR, cNHEJ and aNHEJ), B) SSBR/BER and C) NER. (Image created with BioRender.com)

1.3. Double Stranded Break (DSB) repair

DSB repair is mediated by two distinct pathways; homologous recombination (HR) and non-homologous end joining (NHEJ). HR takes place in the G2/S phase of the cell cycle, because it requires a template for processing DNA breaks (Delacôte and Lopez 2008; Hartlerode and Scully 2009). Whereas NHEJ can occur at any time in the cell cycle and is particularly important in the G0/G1 cell cycle phase as it does not require a template to repair DNA ends (Delacôte and Lopez 2008; Hartlerode and Scully 2009). Although both HR and NHEJ contribute to DSB repair, the two pathways differ greatly in their signaling cascades, repair processes and biological impact on genome stability.

DSB repair with HR is initiated by the recruitment of the Ataxia-Telangiectasia Mutated (ATM) kinase and the MRN complex to the DNA lesion site (Lavin et al. 2015; Stucki and Jackson 2006). ATM is responsible for the phosphorylation and activation of downstream DDR repair factors (Burma et al. 2001; Matsuoka 2007; Shiloh 2014). The recruitment of the MRN complex in HR is enhanced by DNA damage sensing via Poly-ADP-Ribose Polymerase 1 (PARP1) at the DSB site (Haince et al. 2007, 2008). The MRN complex is comprised of three proteins including Meiotic Recombination 11 (MRE11), Rad50 and Nijmegen Breakage Syndrome 1 (NBS1) (Figure 1.1A) (Lammens et al. 2011). Rad50 forms a complex with MRE11 through its ATPase domain and binds to damaged DNA ends (Lim et al. 2011; G. J. Williams et al. 2011). MRE11 possesses endonuclease activity and double-strand-specific 3'-5' exonuclease activity, which are used to trim the damaged DNA ends (Paull and Gellert 1998). NBS1 functions as a signal transducer, which interacts with MRE11 and associates with ATM to recruit ATM to the DSB site (Lee et al. 2003; R. S. Williams et al. 2009). ATM becomes activated and

subsequently phosphorylates many DDR proteins including the complex itself (Lee et al. 2013). The activation of ATM promotes phosphorylation of histone H2AX, known as γH2AX, which recruits Mediator of DNA Damage Checkpoint protein 1 (MDC1) to the complex (Lou et al. 2006; Stewart et al. 2003; Stucki et al. 2005; Stucki and Jackson 2006). The scaffold protein MDC1 with the E3 ligase RNF8, helps to further recruit and maintain associated downstream DDR proteins at the DNA damage site including p53 Binding Protein 1 (53BP1) and breast cancer type 1 susceptibility protein (BRCA1) (Huen et al. 2007; Kolas et al. 2007; Lou et al. 2003; Mailand et al. 2007). The activation of ATM also recruits DNA endonuclease RBBP8 (also known as CtIP) to the MRN complex and promotes BRCA1-CtIP interaction (L. Chen et al. 2008; Yu et al. 2006). The combined action of the ATM-MRN-CtIP-BRCA1 complex guides DNA DSB end resection to produce a long single stranded 3'- overhang, which is then bound and stabilized by Replication Protein A (RPA) (Hartlerode and Scully 2009; Jensen, Carreira, and Kowalczykowski 2010; J. Liu et al. 2010; Sartori et al. 2007; Wright, Shah, and Heyer 2018). The resected DNA end invades into the homologous template located in the sister chromatid. The RAD51/ breast cancer type 2 susceptibility protein (BRCA2) complex mediates the subsequent strand exchange, migration, and various DNA polymerases are recruited to promote new DNA synthesis (Hartlerode and Scully 2009; Jensen, Carreira, and Kowalczykowski 2010; J. Liu et al. 2010; Sartori et al. 2007; Wright, Shah, and Heyer 2018). Lastly, the formed DNA junctions are cut, and the DNA is re-ligated to complete DSB repair by HR (Figure 1.1A) (Hartlerode and Scully 2009; Jensen, Carreira, and Kowalczykowski 2010; J. Liu et al. 2010; Sartori et al. 2007; Wright, Shah, and Heyer 2018). Since HR uses homologous DNA as a template, DSB repair by HR is considered error free and a key mechanism used by the cell to maintain genomic stability (Hartlerode and Scully 2009).

NHEJ is another prominent DDR pathway, which utilizes a different approach to repair DSBs. There are two types of NHEJ pathways, classical (cNHEJ) and alternative (aNHEJ). cNHEJ is initiated by ATM activation, however the pathway recruits Ku proteins (Ku70, Ku80) to the complex at the DNA lesion in order to align the segments for repair (Dobbs, Tainer, and Lees-Miller 2010; Walker, Corpina, and Goldberg 2001). DNA-dependent Protein Kinase catalytic subunit (DNA-PKcs) proteins bind to the Ku complex to recruit and activate the downstream DNA endonuclease Artemis protein for processing and preparation of the DNA ends for repair (Ma et al. 2002). The Ku complex further helps to recruit X-ray Repair Cross-Complementing protein 4 (XRCC4), DNA polymerases and DNA ligases to fill and ligate the DNA ends to complete DNA repair (Figure 1.1A) (Gu et al. 2007; Nick McElhinny et al. 2000). Alternatively, the aNHEJ pathway is also used, and can be mediated by the presence of micro-homologous sequences flanking on either side of DSBs (Truong et al. 2013). aNHEJ is facilitated by the activation of ATM and association of CtIP with the MRN complex to promote the downstream recruitment of X-ray Repair Cross-Complementing protein 1 (XRCC1) (Audebert, Salles, and Calsou 2004; McVey and Lee 2008; Rahal et al. 2010; Rass et al. 2009; H. Wang et al. 2005). XRCC1 recruits a separate set of DNA polymerases (Pol θ) and ligases to fill and re-join the DNA ends (Figure 1.1A) (Audebert, Salles, and Calsou 2004; Kent et al. 2015; McVey and Lee 2008; Rahal et al. 2010; Rass et al. 2009; H. Wang et al. 2005). Therefore, since the NHEJ pathways repair DSBs by joining two broken DNA ends together, this type of repair can lead to subsequent DNA deletions and mutations (Truong et al. 2013). Thus, NHEJ is considered an error-prone repair mechanism associated with genome instability (Truong et al. 2013).

1.4. Single Stranded Break (SSB) repair

Single stranded breaks arise from various sources in the cell that can be both endogenous or exogenous, such as oxidative stress resulting from reactive oxygen species (ROS) and exposure to UV irradiation (Demple and DeMott 2002; Hegde, Hazra, and Mitra 2008). If left unrepaired, the accumulation of SSBs can cause DSBs, which are highly toxic and detrimental to the cell (Kuzminov 2001). SSB repair is mediated by two overlapping pathways with some repair proteins participating in both. In response to single stranded breaks that arise from sugar disintegration or damaged/missing bases, the BER pathway is activated to mediate the repair of short nucleotide gaps in the DNA (Figure 1.1B) (Keith W Caldecott 2008; Frosina et al. 1996). Larger nucleotide gap repair is mediated by the SSBR pathway which typically arises from UV irradiation caused nucleotide cross-linking (Figure 1.1B) (Keith W Caldecott 2008; Frosina et al. 1996). Both pathways use XRCC1 as a scaffold to recruit downstream DDR proteins for SSB repair (Keith W Caldecott 2007). However, recruitment of XRCC1 in the SSBR pathway is dependent on Poly-ADP Ribose Polymerase 1 (PARP1) and its poly-ADP ribosylation activity (PARylation) to synthesize PAR polymers at the SSB lesion site, whereas BER can recruit XRCC1 in a PARP-independent manner utilizing the DNA-(apurinic or apyrimidinic site) endonuclease (APE1) protein (Keith W Caldecott et al. 1996; Keith W Caldecott 2003; Campalans et al. 2013; Dantzer et al. 2000; El-Khamisy et al. 2003; Mol et al. 2000; Okano et al. 2003; Polo et al. 2019; Prasad et al. 2001). XRCC1 in BER repair mediates the recruitment of DNA polymerase β (Pol β), and DNA ligase III in order to mediate gap filling and ligation (K W Caldecott et al. 1994; Keith W Caldecott 2007; Kubota et al. 1996; Marsin et al. 2003; Sobol et al. 2000). Conversely, in larger patch repair by the SSBR pathway, PARP1 and XRCC1 coordinate the recruitment of Pol β, Pol δ, Pol ε, Flap Endonuclease 1 (FEN1), and Proliferating

Cell Nuclear Antigen (PCNA) to fill and repair the DNA gap, and ligation is mediated by DNA ligase I (Fan et al. 2004; Frosina et al. 1996; Levin et al. 2000, 2004; Y. Liu, Kao, and Bambara 2004; Prasad et al. 2000). Therefore, the two pathways work together to allow for quick detection and repair of SSBs in cells in order to prevent further DNA damage accumulation.

1.5. Nucleotide Excision Repair (NER)

NER pathway is most commonly activated in response to DNA damage caused by pyrimidine dimer photoproducts from UV radiation, or other exogenous chemicals that induce formation of DNA adducts (de Boer and Hoeijmakers 2000; de Laat, Jaspers, and Hoeijmakers 1999; Wood 1997). NER can overlap with BER to manage oxidative DNA damage, however NER mainly mediates the excision of larger bulky DNA adducts that distort the DNA helical shape and stability (de Boer and Hoeijmakers 2000; Ciccia and Elledge 2010; de Laat, Jaspers, and Hoeijmakers 1999; Wood 1997). In NER, up to 30 nucleotides can be excised at once, and the excision is mediated by removal of the DNA adduct from the single strand at both ends (de Boer and Hoeijmakers 2000; Ciccia and Elledge 2010; de Laat, Jaspers, and Hoeijmakers 1999; Wood 1997). Moreover, the opposite strand is used as a template in order to mediate DNA gap filling and re-ligation (de Boer and Hoeijmakers 2000; de Laat, Jaspers, and Hoeijmakers 1999; Wood 1997). The general pathway for DNA damage recognition in global genome nucleotide excision repair (GG-NER), involves recognition of the DNA lesion by the DNA repair protein Complementing XP-C cells (XPC) (Figure 1.1C) (Sugasawa et al. 1998; Volker et al. 2001). Recognition of the lesion by XPC recruit's Transcription Factor II H (TFIIH), DNA repair protein complementing XP-A cells (XPA), RPA and DNA Damage-Binding proteins 1 and 2 (DDB1/2) to form a complex that unwinds the DNA and stabilizes the single strand in order to

process DNA excision (Araújo et al. 2000; Araújo, Nigg, and Wood 2001; Evans, Moggs, et al. 1997; Fitch et al. 2003; He et al. 1995; Moser et al. 2005; Sugasawa 2010; Wakasugi et al. 2002). Excision of the DNA from both ends of the lesion is mediated by DNA Excision Repair protein ERCC-1 (ERCC1)/ DNA repair endonuclease XPF (XPF), and the DNA repair protein complementing XP-G cells (XPG) proteins, which are recruited to the site by the upstream DNA repair factors (Evans, Fellows, et al. 1997; O'Donovan et al. 1994; Sijbers et al. 1996; Sugasawa 2010). Lastly, PCNA, various DNA polymerases (Pol δ, Pol ε) and DNA ligase I are added to the DDR complex to mediate gap filling, and re-ligation of the DNA (Figure 1.1C) (Shivji, Kenny, and Wood 1992; Sugasawa 2010).

1.6. PARP1 Mediates PARylation Signaling in DNA Damage

In early response to DNA damage, various PARP proteins have been shown to be recruited to DNA lesions to mediate protein PARylation and to promote downstream DDR protein recruitment (Ray Chaudhuri and Nussenzweig 2017). PARP1 is a nuclear protein and the main Poly ADP-ribose polymerase, which binds to damaged DNA ends and facilitates the addition of PAR (poly ADP-ribose) chains to itself and other acceptor proteins (Figure 1.2B) (d'AMOURS et al. 1999; Kim, Zhang, and Kraus 2005; Ray Chaudhuri and Nussenzweig 2017). PARP1 contains various domains with specific functions in DDR. The zinc finger motifs (ZF1-3) and the tryptophan-glycine-arginine-rich (WGR) domain facilitate PARP binding to DNA, and the central BRCA1 C terminus (BRCT) domain binds PAR, via PARP1 auto-PARylation activity. The conserved catalytic domain (CD) at the C-terminus of PARP1 activates the polymerization of ADP-ribose from the coenzyme NAD^+ in response to DNA damage, forming PAR polymers (Figure 1.2A) (Altmeyer et al. 2009; Dawicki-McKenna et al. 2015; Huambachano et al. 2011;

Langelier et al. 2012; Loeffler et al. 2011; Ray Chaudhuri and Nussenzweig 2017). Functioning as an ADP-ribose transferase and PAR polymerase, PARP1 covalently attaches ADP-ribose to acidic residues (glutamate, aspartate) as well as lysine and cysteine residues of PARP1 and other acceptor proteins (Hassa and Hottiger 2008; N. Li and Chen 2014; Martello et al. 2016; Wei and Yu 2016; Yajie Zhang et al. 2013). The PAR chains are subsequently synthesized on proteins by the continuous addition of ADP-ribose units through 2′,1′′-*O*-glycosidic ribose–ribose bonds to form linear polymers or 2′′,1′ ′′-glycosidic ribose–ribose bonds between ADP ribose units for branched chains (Figure 1.2B) (Alemasova and Lavrik 2019; Kalisch et al. 2012; Kiehlbauch et al. 1993; Rolli et al. 1997; Ruf et al. 1998).

PAR chains on PARP1 can also be non-covalently recognized by various proteins through PAR binding domains, which contributes to signal transduction and regulation of functional activities of PARylated proteins. Many proteins associated in DDR pathways containing PAR binding domains, such as WWE, FHA, BRCT, Macrodomains, PAR Binding Zinc finger (PBZ) domains and PAR Binding consensus Motifs (PBMs) (Ray Chaudhuri and Nussenzweig 2017; Wei and Yu 2016). The recognition and strength of PAR binding depends on number of domains present and binding mode. Many proteins contain multiple domains that can simultaneously bind PAR with greater affinity (Kalisch et al. 2012; Wei and Yu 2016). These domains recognize various portions of PAR chains. PBZ domains can detect linear PAR chains as well as branched ADP-ribose, the BRCT and Macrodomains can bind terminal ADP-ribose units of PAR chains or ADP-ribose monomers, and the FHA and WWE domains can bind iso-ADP ribose in PAR chains (Hou, Chen, and Yu 2019; Kalisch et al. 2012; Wei and Yu 2016). Many DDR proteins contain these binding domains and are able to recognize PAR in order to recruit and scaffold

DNA repair proteins such as XRCC1 (Aberle et al. 2020; Pleschke et al. 2000; Polo et al. 2019). Other proteins such as the histone subunit macroH2A1.1 is able to bind PAR and influence chromatin dynamics during DNA damage (Timinszky et al. 2009). WWE domains are PAR binding domains found on various E3 ligases. Recently RNF146 WWE PAR binding domain has been characterized to mediate ubiquitination of various DNA damage repair proteins including PARP1, XRCC1, DNA ligase III, and KU70, and promotes degradation of PARP1 via ubiquitylation (Kang et al. 2011; Z. Wang et al. 2012). Moreover, these proteins can also become PARylated as well to further influence protein recruitment and scaffolding during DNA damage (Kang et al. 2011; Z. Wang et al. 2012). Therefore, through writing and reading the PAR signals at the DNA lesions, PARP and PAR-binding proteins actively orchestrate DDR signalling, recruitment and chromatin dynamics in response to DNA damage.

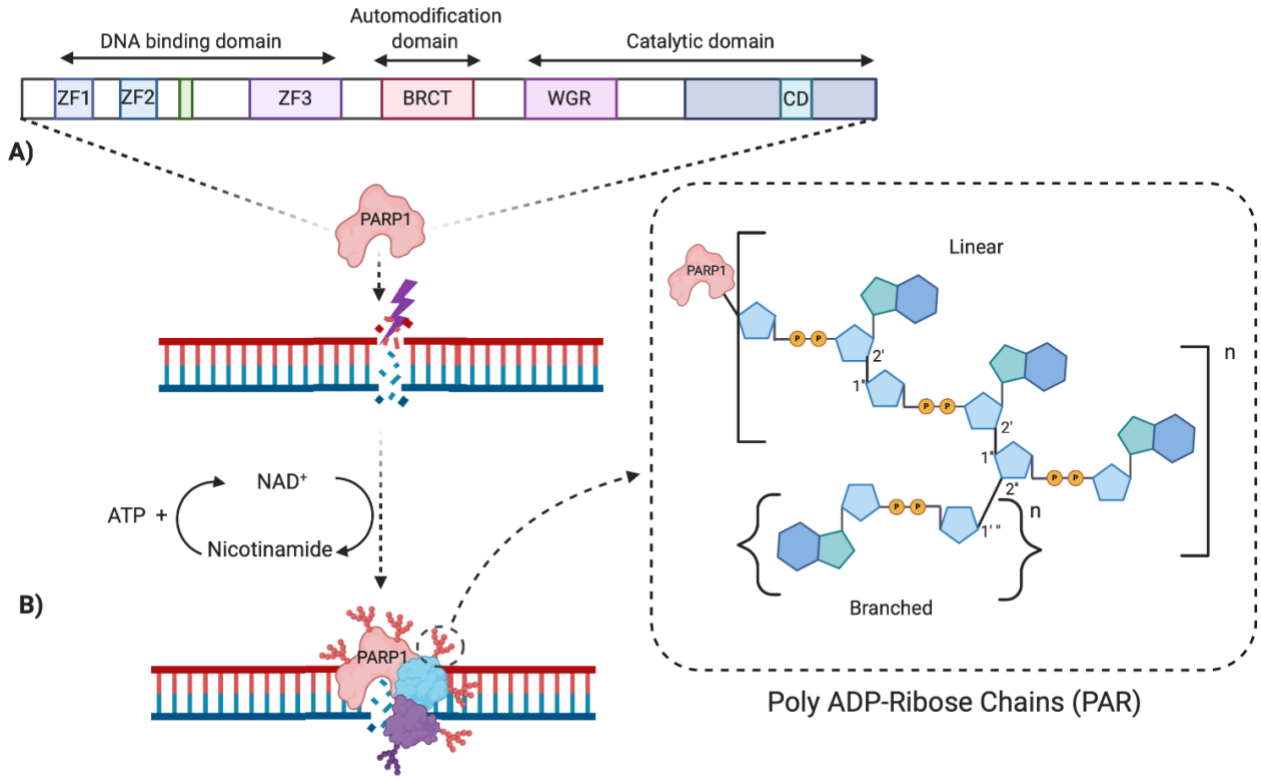


Figure 1.2. PARP1 mediated PARylation in response to DNA damage. A) PARP1 protein domains including zinc finger motifs (ZF1–3), the BRCA1 C terminus (BRCT) domain, and a carboxy-terminal catalytic conserved domain (CD). B) PARP1 binds DNA in response to DNA damage, becomes activated and facilitates PARylation of itself and other proteins. ATP and Nicotinamide are required to replenish NAD^+ as a coenzyme for PARP1 to synthesize PAR chains through polymerization of ADP-ribose. PAR chains on PARP1 and various proteins promote recruitment of various DDR proteins. Figure adapted from: (Ray Chaudhuri and Nussenzweig 2017), and image created with BioRender.com.

1.7. PARP1 Activation in the DNA Damage Response Pathway

PARP1 is conserved within eukaryotes, and has a major role in DNA repair signalling including DSBs and SSBs (Ray Chaudhuri and Nussenzweig 2017). In the DSB repair pathway, PARP1 activity contributes to the recruitment of many early response DNA repair proteins. Many of these proteins contain PAR binding domains, including ATM (PARylation stimulates

ATM activity *in vitro*), MRE11, and BRCA (Haince et al. 2007, 2008; M. Li et al. 2013; M. Li and Yu 2013; Murcia et al. 2001). In response to DNA damage, PARP1 binds DNA lesions, and activates its catalytic activity to promote auto-PARylation, which helps to recruit MRE11, NBS1(MRN complex proteins) and BRCA1 to DNA lesions (Haince et al. 2008; M. Li et al. 2013; M. Li and Yu 2013). PARP1 also influences cellular choice of DSB repair pathways between HR and NHEJ through its PARylation of BRCA1. PARylated BRCA1 associates with Receptor-Associated Protein 80 (RAP80) (PAR binding domain), and the complex is able to downregulate HR by preventing aberrant DSB end processing (Hu et al. 2014). Furthermore, both HR and alternative (a-NHEJ) recruit the MRN complex to facilitate DNA end processing, which is enhanced by PARP1 mediated PARylation (Robert, Dantzer, and Reina-San-Martin 2009; Seol, Shim, and Lee 2018). Moreover, PARP1 coordinates DNA ligase III and X-ray Repair Cross-Complementing protein 1 (XRCC1) to the DNA damage site through PAR binding, to facilitate end-joining (Audebert, Salles, and Calsou 2004; Iliakis 2009). Lastly, *in vitro* studies have shown that PARP1 mediated PARylation of DNA-dependant Protein Kinase catalytic subunits (DNA-PKcs) and promoted kinase activation (Ruscetti et al. 1998; Spagnolo et al. 2012). Therefore, PARP1 promotes activation of DNA-PKcs and KU complex proteins for DNA end repair, signifying its importance in c-NHEJ pathway (Ray Chaudhuri and Nussenzweig 2017).

PARP1 is also involved in SSBR and NER. PARP1 detects single stranded DNA nicks and becomes activated. PARylation of PARP1 on the DNA damage site is recognized by XRCC1 through the BRCT domain and promotes recruitment of other DNA damage repair factors involved in DNA processing and ligation including DNA ligase I, DNA Ligase III, PCNA, and

DNA polymerase β (Dantzer et al. 2000; El-Khamisy et al. 2003; Frouin et al. 2003; Masson et al. 1998; Pleschke et al. 2000; Polo et al. 2019; Wei and Yu 2016). Moreover, in the NER pathway, PARP1 interacts with Xeroderma Pigmentosum group C-complementing protein (XPC), DNA Damage-Binding protein 2 (DDB2) to promote PARylation of various histones in the vicinity of DNA lesions (Ray Chaudhuri and Nussenzweig 2017; Pines et al. 2012; Robu et al. 2013). Histone PARylation promotes recruitment of Amplified in Liver Cancer protein 1 (ALC1) to relax chromatin, in order to further mediate downstream DNA repair proteins to excise damaged DNA caused by UV irradiation (Ray Chaudhuri and Nussenzweig 2017; Pines et al. 2012; Robu et al. 2013). Therefore, PARP1 seems to play an important role in early DDR protein recruitment for a variety of repair pathways, and perturbation of PARP1 could cause deficiencies in DNA damage repair.

1.8. PARP Inhibitors as Cancer Therapeutics

PARP1 regulates and promotes efficient DNA repair in various DDR pathways. Thus, many cancers are sensitive to PARP1 inhibition. PARP inhibitors are able to trap PARP1 to DNA and promote cytotoxic lethality due to replication stress (Michelena et al. 2018; Murai et al. 2014). Moreover, PARP1 inhibitors prevent activation of PARP1 and associated protein PARylation, impeding proper DDR protein recruitment (Bryant et al. 2005; Farmer et al. 2005). Recently, PARP inhibitors have been studied for clinical treatment of BRCA1/2 deficient cancers. Cells with deletion or mutation of BRCA1 and BRCA2 genes often result in genomic instability and are associated with higher risk of breast and ovarian cancer (Bryant et al. 2005; Farmer et al. 2005; Mirza, Pignata, and Ledermann 2018; Noordermeer and van Attikum 2019). In BRCA1 and BRCA2 mutant cancer cells, DSB breaks cannot be repaired via HR, and therefore, cancer

cells rely on alternative DNA repair pathways to survive accumulating DNA damage (Bryant et al. 2005; Farmer et al. 2005). The inhibition of PARP1 promotes synthetic lethality and induces apoptosis in BRCA1/2 deficient cancers (Figure 1.3) (Bryant et al. 2005; Farmer et al. 2005). Loss of PARP1, BRCA1 and BRCA2 activity in DSBs leads to inhibition of DSB repair and results in cancer cell death due to excessive DNA lesions (Bryant et al. 2005; Farmer et al. 2005; Mirza, Pignata, and Ledermann 2018; Noordermeer and van Attikum 2019). Clinical evidence has shown sensitivity of BRCA mutant cancers to PARP inhibitor treatments and supports the effective rationale of using PARP1 inhibitor in treating BRCA deficient cancers (Bryant et al. 2005; Curtin 2012; Farmer et al. 2005; Noordermeer and van Attikum 2019; Rottenberg et al. 2008). However, recent data suggests the treatment is not entirely effective, and that cells can become resistant to PARP inhibitor treatment (Clements et al. 2019; Mirza, Pignata, and Ledermann 2018; Noordermeer and van Attikum 2019). Therefore, identifying proteins involved in the regulation of PARP1 is important to understand the mechanism by which cancer cells vary in their sensitivity to PARP1 inhibitors, and to improve PARP inhibitors as a class of therapeutics for DNA damage repair deficient cancers.

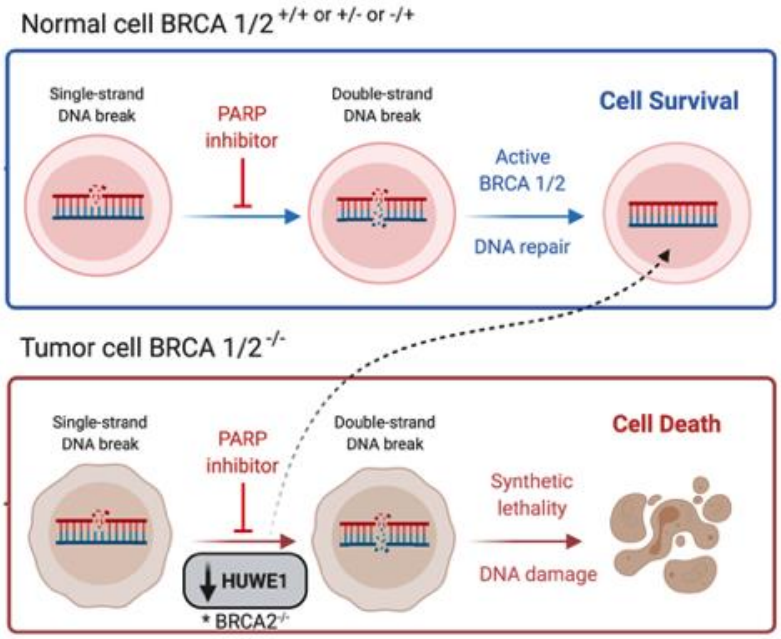


Figure 1.3. PARP inhibitor as a cancer therapeutic in BRCA1/2$^{-/-}$ deficient cells. PARP inhibition causes synthetic lethality in BRCA1/2$^{-/-}$ cells due to improper DNA repair. Knockdown of HUWE1 in BRCA2$^{-/-}$ cells promotes PARP inhibitor resistance and cell survival (Clements et al. 2019) (Created with BioRender.com).

1.9. Ubiquitination in DDR

In order to repair various DNA lesions, the respective DDR pathways need to recruit a myriad of proteins to DNA damage sites. Different post translational modifications of DDR proteins play an important role in protein recruitment to transducing and propagating DDR signals at the DNA lesion. Other than aforementioned protein PARylation and phosphorylation, ubiquitination is another important post translational modification implicated in the cascade of DDR signaling events (Al-Hakim et al. 2010). Ubiquitination of DDR proteins in various DNA repair pathways, could modulate protein stability, activity or scaffolding through a variety of lysine specific modifications to regulate DNA damage repair (Al-Hakim et al. 2010).

Ubiquitination adds ubiquitin (76 amino acid protein) to its substrates by forming an iso-peptide bond between the C-terminus of ubiquitin and the lysine of its target protein (Hershko and Ciechanover 1998; Schwartz and Ciechanover 2009; Pickart 2001). This modification is completed by the sequential actions of the ubiquitin activating (E1), ubiquitin conjugating (E2) and ubiquitin ligating (E3) enzymes respectively (Hershko and Ciechanover 1998; Schwartz and Ciechanover 2009; Pickart 2001). There are three classes of E3 ligases including Ring-Between-Ring (RBR), Really Interesting New Gene (RING) and Homologous to E6AP Carboxyl Terminus (HECT) (Al-Hakim et al. 2010; Scheffner, Nuber, and Huibregtse 1995). RING E3 ligases facilitate the transfer of ubiquitin from the E2 enzyme to the substrate directly, while HECT and RBR E3 ligases form a thio-intermediate with ubiquitin transferred from the E2 enzyme, and then attach the ubiquitin to the substrate (Al-Hakim et al. 2010; Scheffner, Nuber, and Huibregtse 1995). Ubiquitination leads to covalent modification of acceptor proteins with mono-ubiquitin or polyubiquitin chains (Al-Hakim et al. 2010; Tenno et al. 2004). Different forms of ubiquitin chains are synthesized through polyubiquitination with diverse types of linkages. Since ubiquitin has seven lysine residues, each lysine residue could serve as an anchoring point for the polyubiquitination of DDR proteins, which could lead to proteolytic or non-proteolytic effects (Z. J. Chen and Sun 2009; Al-Hakim et al. 2010). Lysine-48 (K48) linked polyubiquitylation is associated with protein degradation by the 26S proteasome, whereas other types of ubiquitylation can regulate proteins through non-proteolytic pathways (Al-Hakim et al. 2010; Hershko and Heller 1985; Pickart 1997; Thrower et al. 2000). Importantly, protein modification by ubiquitin can also be reversed through de-ubiquitinating enzymes (DUBs) that cleave ubiquitin from substrate proteins, which provides another layer of regulation to ensure precise and effective DDR (Le et al. 2019; Sowa et al. 2009).

In DDR, polyubiquitination has been implicated in the recognition and recruitment of DNA damage repair factors. This is exemplified by the role of the E3 ligases RNF8 and RNF168 in promotion of DNA damage signaling through mediating K63 linked polyubiquitylation of histone H2A and H2AX, and other DDR proteins at DNA lesions (Mattiroli et al. 2012; Stewart et al. 2009). RNF8 is recruited to damaged DNA through its forkhead-associated (FHA) domain interaction with phosphorylated MDC1 (Feng and Chen 2012; Kolas et al. 2007). RNF8 binds to the E2 conjugating enzyme Ubc13 to mediate the initial K63 polyubiquitination at the DNA lesion (Feng and Chen 2012; Kolas et al. 2007). DDR signal propagation is further promoted by the subsequent recruitment of RNF168 through its tandem ubiquitin-binding domain (Motif Interacting with Ubiquitin, UIM 1 and 2) to mediate K63 polyubiquitination of H2A and H2AX at the residues K13 and K15 (Doil et al. 2009; Fradet-Turcotte et al. 2013; Huen et al. 2007; Mailand et al. 2007). RNF8/RNF168 also assists in the accumulation of 53BP1 and BRCA1 at DNA damage sites (Doil et al. 2009; Huen et al. 2007; Mailand et al. 2007; Mallette et al. 2012). The amplified K63 chains at the DNA lesion help to hold the associated proteins at the site (Doil et al. 2009; Huen et al. 2007; Mailand et al. 2007). Thus, RNF8 and RNF168 could mediate non-proteolytic signaling through K63 polyubiquitination to promote DDR protein recruitment to DNA lesions in order to mediate efficient DNA repair (Doil et al. 2009; Huen et al. 2007; Mailand et al. 2007; Mallette et al. 2012).

In addition, DDR proteins can be modified by K48-linked polyubiquitination, which signals the targeted proteins for proteasomal degradation. RNF8 has been shown to mediate K48 linked polyubiquitination of NHEJ pathway proteins Ku80 and PCNA to facilitate their removal from chromatin (Feng and Chen 2012; S. Zhang et al. 2008). RNF146 is a RING domain E3

ligase, which selectively interacts with PARylated proteins at DSB sites through its WWE PAR binding domain to promote K48-linked polyubiquitination and proteasomal degradation of PARylated DDR proteins (Kang et al. 2011; Z. Wang et al. 2012).

1.10. HUWE1 in the DNA Damage Response Pathway

HECT, UBA, WWE domain containing 1 (HUWE1) is a HECT-domain E3 ligase, and is known to be involved in ubiquitin mediated degradation and signaling in a variety of cellular processes including apoptosis, DNA replication, and DNA damage and repair (Choe, Nicolae, Constantin, Imamura Kawasawa, Delgado-Diaz, et al. 2016; X. Wang et al. 2014; Zhong et al. 2005). HUWE1 is a large protein (482 KDa) encoded by the *HUWE1* gene on chromosome Xp11.22, and is highly conserved among eukaryotic organisms. (D Chen, Brooks, and Gu 2006; Santos-Rebouças et al. 2015). Although most of HUWE1 is less characterized, several functional domains have been reported (Figure 1.4). Closest to the N-terminus site are the ubiquitin binding domain (UBA) and ubiquitin interaction motif (UIM), which are responsible for recognizing and binding ubiquitin (Z. Liu, Oughtred, and Wing 2005). The WWE domain has been shown to bind iso-ADP ribose *in vitro* (Z. Wang et al. 2012). Moreover, HUWE1 also contains a BH3 domain which binds Bcl-2 associated proteins that are involved in cell death via apoptosis (Zhong et al. 2005). HUWE1 also contains a PCNA-interacting peptide (PIP) motif which interacts with PCNA and contributes to replication fork stability to promote genomic integrity (Choe, Nicolae, Constantin, Imamura Kawasawa, Delgado-Diaz, et al. 2016). Lastly, the C-terminus of HUWE1 contains the enzymatically active HECT domain responsible for HUWE1 mediated ubiquitination (Pandya et al. 2010). The HECT domain contains a catalytic cysteine residue

(Cys4341) that can form a thioester bond with ubiquitin (from E2 conjugating enzymes) in order to directly transfer ubiquitin onto its substrates (Pandya et al. 2010).

Figure 1.4. HUWE1 (HECT, UBA, WWE domain containing 1) functional domain protein structure. From Left: Ubiquitin Binding Domain (UBA) and Ubiquitin Interaction Motif (UIM) recognizes and binds ubiquitin. WWE domain binds iso-ADPR *in vitro.* BH3 domain binds Bcl-2 associated proteins. PCNA-interacting peptide (PIP) motif interacts with PCNA. HECT domain, catalytically active domain: contains a cysteine residue that forms a thioester bond with ubiquitin to transfer ubiquitin directly to substrates. Adapted from (Kao, Wu, and Wu 2018), and image created with Biorender.com.

HUWE1 has been shown to play a complex role in DNA damage signaling, through both proteolytic and non-proteolytic processes. HUWE1 can mediate both K48 and K63 polyubiquitylation, which directs the targeted protein for proteasomal degradation or cell signaling (Kao, Wu, and Wu 2018). In response to DNA damage, HUWE1 modifies a variety of proteins that influence cell cycle arrest or apoptosis including tumor suppressor p53 (p53), Histone deacetylase 2 (HDAC2), Induced myeloid leukemia cell differentiation protein 1 (Mcl-1), Cell division control protein 6 homolog (CDC6) and PCNA (Delin Chen et al. 2005; Choe, Nicolae, Constantin, Imamura Kawasawa, Delgado-Diaz, et al. 2016; Hall et al. 2007; J. Zhang et al. 2011; Zhong et al. 2005). Notably, HUWE1 is known to ubiquitinate and degrade p53, which is an important signaling protein that regulates cell responses to genotoxic stress and DNA damage (Delin Chen et al. 2005; Qi et al. 2012). Furthermore, HUWE1 also mediates HDAC2 ubiquitination and degradation, to promote p53 acetylation and subsequent apoptosis in response to DNA damage (J. Zhang et al. 2011). The BH3 domain of HUWE1 associates with another

substrate (Mcl-1), which coordinates its degradation in response to DNA damage and promotes cellular apoptosis (Zhong et al. 2005). CDC6 is another HUWE1 substrate that is involved in replication and G1 checkpoint regulation (Hall et al. 2007). Ubiquitination of CDC6 by HUWE1 influences cell cycle checkpoint arrest to prevent damaged DNA from being further replicated (Hall et al. 2007). Lastly, HUWE1 interacts with PCNA at stalled replication forks, and mono-ubiquitinates H2AX to promote recruitment of repair proteins in order to mitigate replicative stress and promote replication fork restart (Choe, Nicolae, Constantin, Imamura Kawasawa, Delgado-Diaz, et al. 2016). Therefore, HUWE1 regulates the process of cell cycle arrest or cell death in response to DNA damage through ubiquitination of various proteins in the DDR pathway.

Although HUWE1 ubiquitylation has been primarily associated with proteins involved in apoptosis and cell cycle arrest, the role of HUWE1 in the DDR pathway has been well demonstrated. HUWE1's substrate proteins H2AX, ATM, Histone H1, BRCA1, Polβ and Polλ are all signaling proteins and repair enzymes that actively participate in HR, NHEJ, NER and BER respectively (Atsumi, Minakawa, Ono, Dobashi, Shinohe, Shinohara, Takeda, Takagi, Takamatsu, Nakagama, et al. 2015; Kao, Wu, and Wu 2018; Mandemaker et al. 2017; Parsons et al. 2009; X. Wang et al. 2014). It has been shown that HUWE1 regulates basal levels of H2AX via polyubiquitination mediated degradation under unstressed conditions (Atsumi, Minakawa, Ono, Dobashi, Shinohe, Shinohara, Takeda, Takagi, Takamatsu, and Nakagama 2015). The association of ATM and SIRT6/SNF2H with HUWE1 upon DNA damage (DSBs) prevents the ubiquitylation and degradation of H2AX (Atsumi, Minakawa, Ono, Dobashi, Shinohe, Shinohara, Takeda, Takagi, Takamatsu, Nakagama, et al. 2015). Therefore, H2AX becomes

stabilized and incorporated into the chromatin to be further activated by phosphorylation (Atsumi, Minakawa, Ono, Dobashi, Shinohe, Shinohara, Takeda, Takagi, Takamatsu, Nakagama, et al. 2015). HUWE1 has also been shown to ubiquitinate Histone1.2 (H1.2) *in vitro* and more recently found to ubiquitylate H1.2 in UV irradiated cells, which influences downstream DDR recruitment in both DSB repair and NER (Mandemaker et al. 2017). BRCA1 is an important regulator in HR mediated repair of DSBs. Studies have found that HUWE1 negatively regulates BRCA1 in breast cancer cells, and plays a role in suppression of HR to favour NHEJ in response to DSBs (X. Wang et al. 2014). Moreover, the association of HUWE1 with DDR proteins was further demonstrated in a study using HUWE1 deficient MEF and BMKO (*Mule* deleted specifically in B cells) B cells (Hao et al. 2012). In response to HUWE1 depletion under genotoxic stress (doxorubicin), there was a decrease in protein levels of phosphorylated-ATM(Serine1987), phosphorylated-p53 and BRCA1, which are all associated in a variety of DDR pathways (Hao et al. 2012). HUWE1 also regulated levels of Polβ and Polλ through proteolytic ubiquitylation, which is important in BER. Polβ and Polλ regulate gap-filling under DNA damage, therefore improper protein levels of both can lead to aberrant DNA repair and carcinogenesis (Markkanen et al. 2011, 2012; Parsons et al. 2009). Thus, HUWE1 regulates a variety of DDR proteins through both proteolytic and non-proteolytic mediated ubiquitylation signaling.

1.11. Rationale

In order to maintain genomic integrity, the cell has adapted a complex signalling response through the DDR pathway to alleviate DNA damage, promote proper replication and prevent carcinogenesis (Ciccia and Elledge 2010). The DDR pathway is highly intricate and tightly regulated to ensure effective repair of different types of endogenous or exogenous DNA damages. Various pathways such as HR, NHEJ, BER, NER are activated in response to different types of DNA damage including DSBs and SSBs (Ciccia and Elledge 2010). DSBs are highly toxic and lethal to cells if left unrepaired, therefore the mechanism of repair for this pathway is important to understand (Ciccia and Elledge 2010).

HUWE1 is an E3 ligase, previously shown to be involved in DDR by regulating H2AX, ATM, H1, and BRCA1 through either proteolytic or non-proteolytic ubiquitin signalling (Atsumi, Minakawa, Ono, Dobashi, Shinohe, Shinohara, Takeda, Takagi, Takamatsu, Nakagama, et al. 2015; Kao, Wu, and Wu 2018; Mandemaker et al. 2017; X. Wang et al. 2014). However, the role of HUWE1 in the regulation of DDR pathways is not well understood. Apart from the E3 ligase activity of HUWE1 mediated by the HECT domain, the WWE domain of HUWE1 was recently shown to bind ADP ribose *in vitro (unpublished data),* a subunit of PAR. WWE domains have shown to be associated to PARylation mediated signalling through binding Poly ADP-ribose (PAR) chains (Z. Wang et al. 2012). Moreover, a recent study established that the WWE domain containing E3 ligase RNF 146 mediates ubiquitylation of PARylated proteins including PARP1, XRCC1, DNA ligase III and KU70 at the DNA damage site (Kang et al. 2011). The ubiquitination activity of RNF146 is promoted by the interaction between its WWE domain and the PAR chains attached on the targeted proteins associated in DNA damage repair

(Kang et al. 2011). PARP1 has been shown to be the primary nuclear PAR polymerase and plays an important role in DDR protein recruitment in both DSBs, SSBs, and NER (Ray Chaudhuri and Nussenzweig 2017). Although HUWE1 contains a WWE domain, it is not known whether HUWE1 interacts with PAR and PARP1. Moreover, despite the fact that both HUWE1 and PARP1 are involved in DDR signalling, the functional relationship between the proteins in DDR has not been characterized.

A recent study has shown that knockdown of HUWE1 in BRCA2 deficient cells promoted PARP1 inhibitor resistance (Figure 1.3) (Clements et al. 2019). Therefore, it would be important to understand the functional association between HUWE1 and PARP1 in order to improve the understanding of the pathological role of HUWE1 in DDR deficient cancers. Based on prior studies of HUWE1 in DNA repair, the hypothesis of the study is that HUWE1 is functionally associated with DNA repair proteins in the DDR pathway. Moreover, since HUWE1 contains a WWE domain, HUWE1 could interact with PAR or PARP1 through its WWE domain. Thus, the aims of the study include the following:

1. *Characterizing the role of HUWE1 in the DDR pathway*

   Although HUWE1 associates with various proteins in the DDR pathway, the relationship between HUWE1 and DDR proteins in response to DNA damage is not well characterized. Experiments were performed to detect the effect of HUWE1 perturbation on associated DDR proteins in response to DNA damage, to better understand how HUWE1 functions in the DDR pathway.

2. *Determining the interaction of HUWE1 with PAR and PARP1*

   HUWE1 contains a WWE domain and binds ADP ribose *in vitro.* Therefore, the interaction of HUWE1 with PAR and PARP1 was evaluated in HEK 293T cells. Cells overexpressed with FLAG-tagged HUWE1 were immunoprecipitated using PAR antibody and immunoblotted for HUWE1 in order to determine interaction. Moreover, cells overexpressed with FLAG-tagged HUWE1 were immunoprecipitated with FLAG antibody and immunoblotted for PARP1 in order to determine interaction.

3. *Determining HUWE1 as an E3 ligase mediating PARP1 ubiquitination*

   In order to determine if the stability of PARP1 was associated with HUWE1 mediated ubiquitylation, a ubiquitylation assay using stably knocked down HUWE1(shRNA) in U2OS cells was performed. Cells were treated with a proteasome inhibitor (MG132) and immunoprecipitated using PARP1 antibody, to evaluate PARP1 ubiquitylation.

The overall goal of this study is to improve the understanding of the role of HUWE1 in the regulation of DDR pathways. By studying the association of HUWE1 with DDR protein PARP1 and PAR, this study would provide better understanding of the DDR signaling through protein ubiquitination and PARylation.

Chapter 2: Materials and Methods

2.1 Cell Culture

Human Embryonic Kidney cells (HEK 293T) cells were kindly donated from Dr. Peng's laboratory 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 FLAG-tagged HUWE1 (H) and vector (V) cells were donated from Dr. Mak's laboratory and were similarly cultured in DMEM media supplemented with 10% FBS, 1% P/S, and 70μg/ml of geneticin (G418, *Gibco Cat#10131-035*) in order to retain cells expressing the selected plasmids (Dominguez-Brauer et al. 2017). Moreover, Mouse Embryonic Fibroblasts (MEF) cells donated from Dr. Mak's laboratory were also cultured in DMEM media supplemented with 10% FBS and 1% P/S (Hao et al. 2012). BJ Fibroblast, and Osteosarcoma (U2OS) cells were donated from Dr. Benchimol's laboratory and were cultured in DMEM, and McCoy's 5A modified medium (*Life Technologies Cat# 16600082*) respectively, and also supplemented with 10% FBS and 1% P/S. Lastly, shHUWE1-F6 and shHUWE1-G10 cells used to study the knockdown effects of HUWE1,were cultured in McCoy's 5A modified medium supplemented with 10% FBS, 1% P/S and 1-3μg/ml of puromycin (*Gibco Cat# A11138-03*) in order to maintain cells expressing the shRNA plasmids.

2.2 Cell Treatments

In order to induce UV radiation damage, media was removed from cells and rinsed with 1X phosphate buffered saline (PBS, *Life Technologies Cat# 14190144*). Open plates were placed into a UV crosslinker (*SpectroLinker XL-1500*), and the device was programmed on the J/m^2 energy setting in order to irradiate the cells with the desired dose of UV light. The dosage

experiment utilized various doses of UV radiation (10-100 J/m^2), whereas experiments afterwards were optimized and treated with 20 J/m^2 of UV radiation.

In order to induce DSBs, a topoisomerase II inhibitor (Etoposide, *Sigma Cat# E1383*) was used in cell treatment. In the dosage experiment various concentrations of etoposide (1-20μM) were used, however experiments optimized afterwards were treated with 10μM of the drug. Moreover, since the etoposide stock was dissolved in dimethyl sulfoxide (DMSO, *Sigma Cat# D-2650*), the vehicle control samples were supplemented with the equivalent volume of DMSO in the experimental treatments.

2.2.1. Time Course Conditions

The cell treatments previously mentioned were used to survey DNA damage over various time points between 0 minutes- 4hours. UV radiation and drug treatment were initially applied to the cells and then cells were harvested at specific time points after treatment. The controls including untreated samples, and samples containing DMSO were harvested using trypsin (*Life technologies Cat# 25300054*) at the end of the last timepoint.

2.3 Cell Lysis

Cell lysis procedures were kindly contributed by Dr.Cheung's laboratory and slightly modified.

2.3.1. Total Cell Lysate

Cell pellets were resuspended in 250-500μl of total cell lysis (TCL) buffer with appropriate inhibitors (Table 2.1 and Table 2.2) in order to permeate and breakdown the cell membrane. Inhibitors were added to prevent degradation of associated protein signals and

complexes. Cell lysis was further achieved through sonication of the lysate (20% Intensity, Duty Cycle 3) for 90-100 pulses. Samples were spun at 2100rpm at 4°C for 5 minutes to pellet the cell debris, and the supernatant containing the cell proteins was aliquoted separately. A BCA assay (*ThermoFisher Scientific Cat#23225*) with bovine serum albumin (BSA) protein standards was used to create a standard curve in order to measure the unknown samples protein concentrations. Specific concentrations of samples were prepared to ensure equal protein loading for western blot analysis. Furthermore, samples were prepared with sodium dodecyl sulfate (SDS) and boiled for 5 minutes in order to further denature proteins and were frozen at -20°C until further immunoblot processing.

Table 2.1. Total Cell Lysis (TCL) Buffer Recipe

Reagent	Stock Concentration	Working Concentration
Tris pH7.4	1M	20mM
Ethylenediaminetetraacetic acid (EDTA)	0.5M	20mM
Non-ionic Polyoxyethylene Surfactant (NP40)	10%	1%
Glycerol	50%	20%
NaCl	1M	400mM

Table 2.2. Supplemental Protease Inhibitors

Reagent	Stock Concentration	Working Concentration
N-Ethylmaleimide (NEM)	400X	1mM
Phenylmethane Sulfonyl Fluoride (PMSF)	100µM	0.2µM
Benzamidine Hydrochloride (Ben HCl)	1M	1mM
Protease Cocktail Inhibitor (PI) (Roche Cat# 5056489001)	50X	1X
PARG-inhibitor (ADP-HPD) (Millipore Cat# 118415)	100µM	1µM
Phosphatase Inhibitor (ThermoFisher Scientific Cat#78428)	100X	1X

2.3.2. Nuclear Fractionation

Cell pellets were resuspended in 500-1000µl of nuclear extraction buffer with appropriate inhibitors (Table 2.2 and Table 2.3) in order to permeate and breakdown the outer cell membrane. Inhibitors were added to prevent degradation of associated protein signals and complexes. Samples were spun at 2100rpm at 4°C for 5 minutes to pellet the nuclear fraction, and the supernatant containing the cytoplasmic proteins was aliquoted separately. The nuclear fraction was resuspended in 2X SDS buffer (Table 2.4) and sonicated (20% Intensity, Duty Cycle 3) for 90-100 pulses to ensure proper nuclear envelope lysis. A BCA assay (*ThermoFisher Scientific Cat#23225*) with bovine serum albumin (BSA) protein standards was used to create a standard curve in order to measure the unknown samples protein concentrations. Specific concentrations of samples were prepared to ensure equal protein loading for western blot analysis. Furthermore, samples were prepared with sodium dodecyl sulfate (SDS) and boiled for 5 minutes in order to further denature proteins and were frozen at -20°C until further immunoblot processing.

Table 2.3. Nuclear Extraction Lysis Buffer Recipe

Reagent	Stock Concentration	Working Concentration
Tris pH 7.6	1M	10mM
NaCl	1M	150mM
$MgCl_2$	1M	1.5mM
Non-ionic Polyoxyethylene Surfactant (NP40)	10%	0.65%

Table 2.4. 2X SDS Lysis Buffer Recipe

Reagent	Stock Concentration	Working Concentration
Tris pH 7.4	1M	20mM
Ethylenediaminetetraacetic acid (EDTA)	0.5M	20mM
Sodium Dodecyl Sulfate (SDS)	10%	2%
Glycerol	50%	20%

2.4 Immunoblotting

Prepared lysate samples stored at -20°C, were thawed at room temprature and re-boiled for 5 minutes to ensure protein denaturation. Samples were equally loaded on 4-12% gradient gels (*ThermoFisher Scientific Cat# XP04120*) with a stained protein ladder (*ThermoFisher Scientific Cat# 26616*). The SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) gels were run at 120-130 volts (V) for approximately 1-1.5hours. The acrylamide gels were transferred onto methanol activated PVDF membranes and transferred overnight at 4°C at 30V to ensure proper and complete transfer of both large and small proteins.

After the transfers were completed the immunoblots were stained with Ponceau S (*Santa Cruz Cat# sc-301558*) for 5 minutes to ensure proper protein transfer. The blots were then blocked with 5% milk in phosphate buffered saline with Tween 20 (PBST) or TBST for 1 hour. The immunoblots were then incubated with appropriate primary antibodies (prepared accordingly to Table 2.5) overnight at 4°C, to ensure proper protein-antibody binding for signal detection. Membranes were then washed with PBS and incubated with the species appropriate secondary HRP antibodies in order to bind the primary antibodies (Table 2.5). After a 1-hour incubation, the blots were washed, and the secondary antibodies HRP enzyme activity was activated using Western Lightning Plus-Enhanced Chemiluminescence Substrate (ECL) (*PerkinElmer Cat# NEL105001*). The immunoblots were visually detected using the MicroChemi digital imager (*DNR Bio-Imaging Systems*), and the associated image files were processed using ImageJ version 2.0.0.

Table 2.5. List of Antibodies and Associated Dilution Preparations

Antibody Name	Manufacturer	Catalogue No.	Species	Dilution	Dilution Solvent (in PBST or TBST)
HUWE1	Bethyl	A300-486A-M	Rabbit	1:1000 µl	5% Milk
γH2AX(Ser139)	CST	80312	Mouse	1:1000 µl	5% BSA
PARP	CST	9542S	Rabbit	1:1000 µl	5% BSA
PARP1 (HC2R8)	Invitrogen	14-6667-82	Mouse	1:1000 µl	5% BSA
PAR (E6F6A)	CST	83732S	Rabbit	1:1000 µl	5% BSA
Anti-PAR (Ab-1) (10H)	Trevigen	4335-MC-100	Mouse	1:1000 µl	5% BSA
GAPDH	Santa Cruz	sc-47724	Mouse	1:5000 µl	5% BSA
Lamin A/C	Santa Cruz	sc-376248	Mouse	1:1000 µl	5% Milk
Lamin B2 (D8P3U)	CST	12255	Rabbit	1:1000 µl	5% BSA
Phospho-NBS1 (Ser343)	CST	3001	Rabbit	1:1000 µl	5% BSA
Ubiquitin (P4D1)	Santa Cruz	sc-8017	Mouse	1:1000 µl	5% BSA
Phospho-ATM (Ser1981) (D6H9)	CST	5883	Rabbit	1:1000 µl	5% BSA
Phospho-ATM (Ser1981) C.70.6	Invitrogen	MA5-15185	Mouse	1:1000 µl	5% BSA
Anti-PAR (Ab-1) (10H)	Millipore/Calbiochem	AM80-100UG	Mouse	1:1000 µl	5% BSA
H3	CST	14269	Mouse	1:1000 µl	5% BSA
H2AX	CST	2595	Rabbit	1:1000 µl	5% BSA
Alexa 555 (IF)	Invitrogen	A32732	Rabbit	1:1000 µl	5% BSA
Anti-β-Actin	Sigma	5441	Mouse	1:5000 µl	5% BSA
Anti-Rabbit-HRP	Jackson Immuno	711-035-152	Donkey	1:5000 µl	1% Milk
Anti-Mouse-HRP	Jackson Immuno	715-035-150	Donkey	1:5000 µl	1% Milk
Anti-FLAG M2	Sigma	F1804	Mouse	1:1000 µl	5% BSA
DAPI	Invitrogen	R37606	Drops	2 drops/ml	N/A
* Bovine Serum Albumin (BSA)					

2.5 siHUWE1 Knockdown

In order to study the effect of HUWE1 knockdown on DDR proteins, U2OS cells (10cm plates at 75% confluency) were transfected according to the Lipofectamine RNAiMAX established protocol (*ThermoFisher Scientific Cat# 13778075*). 10µl (20µM stock) of HUWE1 siRNA (AAUUGCUAUGUCUCUGGGACA) (*Genepharma ARF-BP1 Lot 657 1:00D duplex*) was added to 500µl of OptiMEM media or 10µl (20µM stock) of control siRNA (*CST Cat# 6568S*) was added to 500µl of OptiMEM media (Dominguez-Brauer et al. 2017). 5µl of lipofectamine reagent (*ThermoFisher Scientific Cat# 13778075*) was added to 495µl of OptiMEM media (x2) and was prepared separately. The diluted siRNAs were added to the lipofectamine reagents in a 1:1 ratio. The mixtures were pipetted up and down and incubated at room temperature for 5 minutes before being added to the U2OS cells (1ml). The cells were supplemented with an additional 9ml of McCoy 5A medium 4 hours afterwards, and then incubated for 16-20 hours at 37°C with 5% CO_2. The media was then replaced after the 16-20hour incubation. 48 hours after transfection, the HUWE1 knocked down cells were treated with 10µM of etoposide for 1hour to induce DNA damage. The cells were harvested and stored -80°C, until pellets were processed for immunoblotting as described above.

2.6 shHUWE1 Cell Line Development

2.6.1. 293T Lentiviral Particle Formation

The lentiviral protocol was adapted from the PolyJet mammalian cell transfection protocol (*SignaGen Cat# SL100688*). 293T cells were incubated with an equal ratio of shHUWE1-G10, shHUWE1-F6 plasmid DNA (6µg) and lentiviral packaging DNA (3µg VSVG and 3µg PAX2) (Inoue et al. 2013). The DNA mixtures were added to serum free DMEM media

and diluted PolyJet reagent was added to promote the DNA complex entry into the cell. Cells were incubated with the DNA mixture overnight at 37°C with 5% CO_2, and then replaced with fresh DMEM media. The lentiviral particles in the supernatant were harvested 48-72 hours post transfection and filtered through a 0.22μm membrane filter syringe to remove any cell debris.

2.6.2. U2OS Viral Infection

U2OS cells were grown in 6 cm dishes with McCoy 5A medium supplemented with 10% FBS and 1% P/S. In order to infect the cells, the McCoy media was replaced with 1ml of RPMI media, 1.44μl of polybrene (*Sigma Cat# 28728-55-4*) and 1ml of shHUWE1-F6 or shHUWE1-G10 lentiviral particles and incubated for 24-48 hours to allow cell infection. A U2OS kill curve assay was performed to determine puromycin antibiotic concentration used for cell selection. U2OS cells were found to be sensitive to treatment (concentrations ranging between 1-3μg/ml) over the course of 3-5 days. Therefore, after lentiviral infection the shHUWE1-G10 or shHUWE1-F6 cell media was replaced with McCoy 5A medium supplemented with 1-3μg/ml of puromycin. Since, shHUWE1 cells that were transfected carried a puromycin resistance gene, they were selectively grown in puromycin supplemented media. Moreover, since the shHUWE1 plasmids also contained a GFP marker, GFP expression of shHUWE1-F6 and shHUWE1-G10 cells were validated under a microscope and immunoblotted for HUWE1 depletion in order to check the lentiviral transfection efficiency.

2.7 Immunofluorescence Staining and Detection

Wild type U2OS, shHUWE1-F6 and shHUWE1-G10 cells, were seeded on coverslips (50-80% confluent) and treated with etoposide (10μM) or DMSO as a control for 1 hour. Coverslips were washed with PBS after treatment, and 0.5ml of 1% paraformaldehyde in PBS (filtered) was added onto coverslips to fix the cells. Afterwards, 0.5% TritonX in PBS (filtered) was added in order to permeate the cells. The cells were then washed with PBS and 5% Bovine Serum Albumin (BSA) in PBST was added for 1 hour. The PARP1 primary antibody was added and incubated overnight at 4°C. Afterwards, the slides were washed and incubated with fluorescent secondary antibody (Alexa 555) in order to visualize PARP1 (Table 2.5). Moreover, DAPI drops were additionally added to the slides in order to stain the nucleus, and slides were mounted with antifade (*ThermoFisher Cat# P36961*). The slides were left overnight to dry and later sealed with clear nail polish and stored in a dark box at 4°C to prevent fluorescent signal degradation. The slides were later visualized with the Zeiss Laser Scanning LMS 700 Confocal Microscope at 20X objective, and the images obtained were processed using ImageJ version 2.0.0.

2.8 Immunoprecipitation Assays

Cell pellets were resuspended in 500μl of IP lysis buffer (Table 2.6) and supplemented with PMSF, BenHCl, PI, and PARG-inhibitor (Table 2.2). The resuspended cells were incubated on ice and periodically vortexed for 20 minutes to promote cell lysis. Cells were then sonicated (10% intensity and Duty cycle 3) in 30 pulse intervals (3X) to avoid over-heating the samples. The cell lysates were spun down at 13000rpm for 10-15 minutes at 4°C to remove cell debris, and the supernatant was separately aliquoted. The supernatant was subjected to a BCA assay to measure protein concentration, and approximately 2mg of protein was used to set up the

immunoprecipitation reaction with respective antibodies. 500µl reactions were set up with cell lysate (2mg of protein), and primary antibody (used to pull down desired protein, between 2-4µl) and incubated at 4°C for 2 hours to facilitate target protein-antibody binding. Moreover, input samples (50 µg) were harvested from the cell lysate, prepared with SDS dye and boiled for 5 mins for western blot analysis later to confirm protein presence. A/G beads (50µl beads/sample) (*Santa Cruz Cat# sc-2003*) were prepared by removing storage buffer and precleared with 5% BSA in PBST or cell lysate for 30 minutes. A/G beads were then spun down (2000rpm, 5 minutes, 4°C) and reconstituted in the original IP lysis buffer and added to the immunoprecipitation reaction for 1 hour at 4°C to promote complex binding to the beads. After incubation, the lysate/bead mixture was spun down (2000rpm, 5 minutes, 4°C) and washed 5 times with lysis buffer (in 10-minute increments) to remove unspecific protein binding. After the last wash, the mixture was spun down, the supernatant was removed and 50µl of 2X SDS dye was added. The samples were stored at -20°C, and prior to immunoblot processing the samples were boiled for 5 minutes to denature the proteins further and spun down so that the supernatant could be used for the SDS-PAGE gel. Samples were immunoblotted afterwards as described previously.

Table 2.6. IP Lysis Buffer Recipe

Reagent	Stock Concentration	Working Concentration
Tris pH 7.4	1M	50mM
NaCl	1M	50mM
Non-ionic Polyoxyethylene Surfactant (NP40)	10%	1%
Glycerol	50%	10%

2.9 PARP1 Ubiquitination Assay

In order to determine HUWE1's role in PARP1 ubiquitylation, a ubiquitylation assay was performed with wild type U2OS and shHUWE1-G10 (HUWE1 knockdown) cells. Cells were grown to 75-95% confluency in 10cm plates, and fresh McCoy media was supplemented with either DMSO as a control or 5μg/ml of MG132 (*Calbiochem Cat# 474787-10MG*) for 4 hours to allow proteasome inhibition. After the 4-hour incubation the cells were harvested, and stored at -80°C, until processed for an immunoprecipitation with PARP1 antibody as described above. PARP1 ubiquitylation was evaluated by immunoblotting with ubiquitin antibody against PARP1 immunoprecipitants.

Chapter 3: Results

Experiments were designed by Gabriella Gerzon and Dr. Yi Sheng and performed by Gabriella Gerzon, and contributions to some of the experiments were made by Chetna Raj, Miriam Basta, and Greta Raffoul as part of their undergraduate research training

HUWE1 is a protein shown to be involved in DNA damage and cellular regulation, however how HUWE1 is involved in the DDR pathway through the regulation of histones and DNA damage repair proteins is still largely unknown. In order to understand HUWE1's role in the DDR pathway, cellular response to different DNA damage upon HUWE1 perturbation were examined.

3.1 Cellular Responses to UV and Etoposide Induced DNA Damage

To determine appropriate cell treatment to induce DNA damage, a dosage experiment using HEK 293T cells treated with various DNA damage agents was performed. DNA damage specific histone γH2AX was surveyed in order to measure the cellular response to treatment and to evaluate the associated DNA damage induced. Cells were subjected to two types of DNA damage agents; etoposide and UV in order to survey responses to different DNA damages induced. Treatment with etoposide, a topoisomerase II inhibitor, induced DSBs, which is associated with HR and NHEJ DNA repair (Rassool 2018). Treatment conditions with increasing etoposide concentrations between 1-20μM were used to evaluate H2AX activation by phosphorylation in response to induced DNA damage. Moreover, UV could induce both SSBs and DSBs depending on the doses of UV irradiation. Treatment of 10-100 J/m^2 was used to investigate histones H2AX phosphorylation (γH2AX) in response to UV induced (SSBs and DSBs) DNA damage. As expected, accumulation of γH2AX was observed in cells treated with etoposide and UV, compared to the untreated (UT) controls (Figure 3.1). Robust levels of γH2AX were seen from 20-100 J/m^2 of UV treatment and 10-20μM of etoposide treatment, and therefore further experiments in this study used this range of treatment to induce DNA damage. Moreover, in addition to accumulation of γH2AX in response to the DNA damage, which

migrated at 15 KDa, higher molecular weight species between 20 and 35 KDa were also seen for H2AX as well as γH2AX immunoblots, indicating there may also be other forms of post-translational modifications to these histones in response to DNA damage.

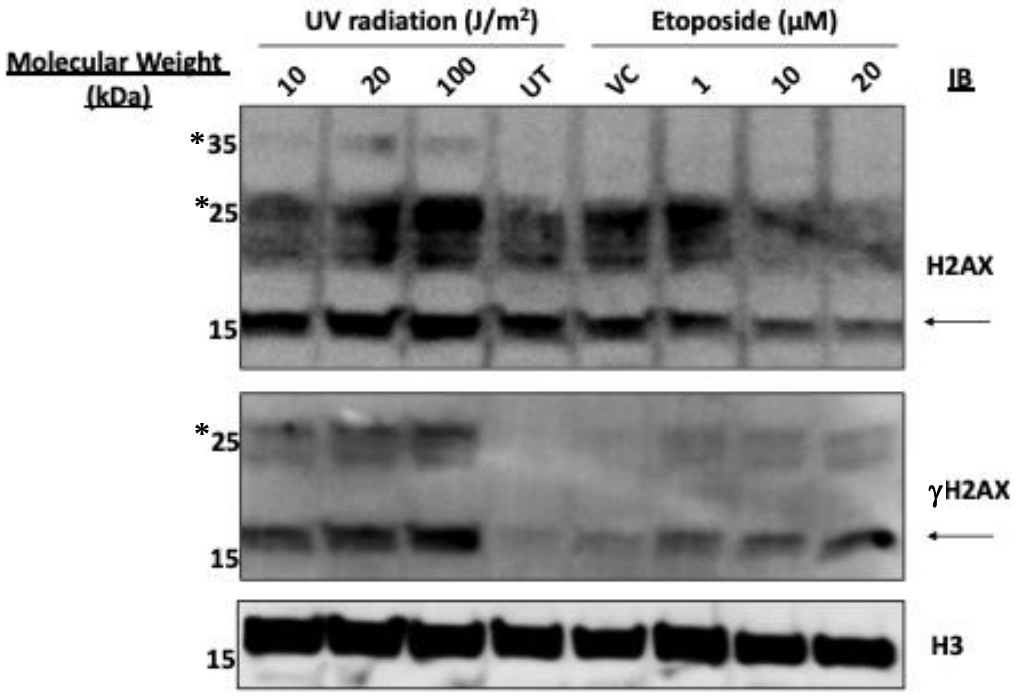


Figure 3.1. DDR Histone Response to DNA Damage: Dosage Experiment. HEK 293T cells treated with Etoposide (1-20 μM) and UV (10-100 J/m^2) radiation treatment for 4 hours and harvested. 20μg of nuclear extract subjected to Immunoblot visualization using H2AX(Cat#:2595), γH2AX (Cat#:80312), and H3 (Cat#:14269) as a loading control. UV=J/m^2. (UT) represents untreated sample, and (VC) represents vehicle control containing 5ul of DMSO. (*) represents modified H2AX and γH2AX histones.

Further experiments were performed in order to determine DNA damage induced γH2AX response in different cell lines over specified time periods with both etoposide and UV treatment. In HEK 293T cells, γH2AX was detected as early as 30 minutes into treatment with respective DNA damage agents and accumulated throughout the entirety of the time course (2-4 hours) (Figure 3.2). This was also confirmed in another cancer cell line (U2OS) and normal fibroblasts cells (BJ) with both UV and etoposide treatment (Supplementary Figure 1A and 1B). Using γH2AX as a DDR marker, these results collectively showed that the cellular DDR response to DNA damage induced by etoposide and UV occurred early and accumulated over the time course studied. Therefore, further experiments were modelled with the same cell treatment conditions to induce DNA damage and time course periods to survey DDR activation in response to DNA damage.

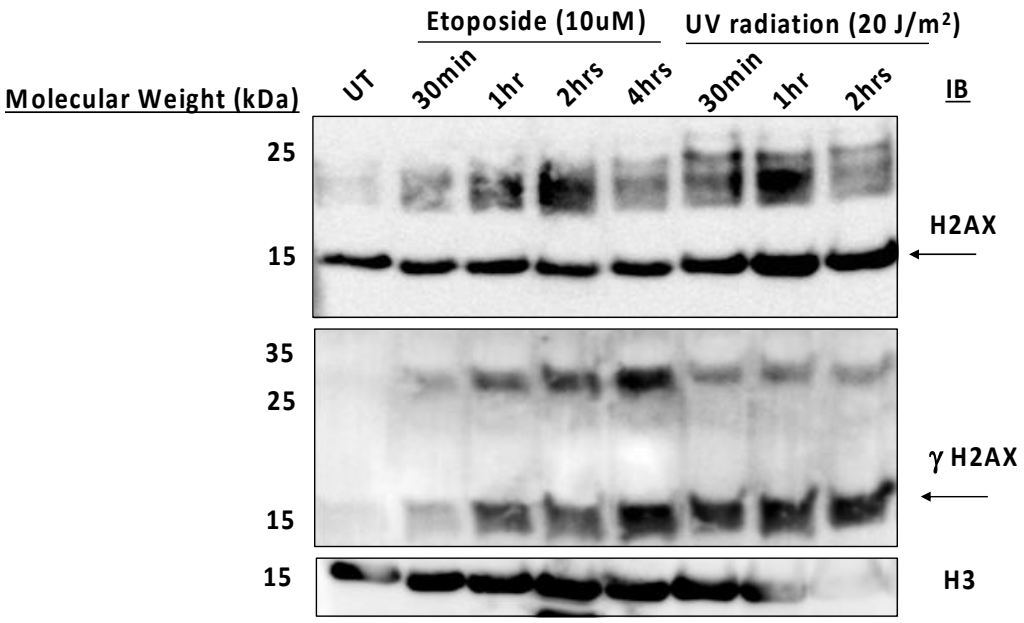


Figure 3.2. Histone Response to DNA Damage: Time-Course Experiment. Histone activation and various modifications in HEK 293T cells under induced DNA damage. Cells were treated with Etoposide (10μM) and UV radiation (20J/m^2) treatment for 30min-4 hours. Immunoblot visualization using 20μg of nuclear extract with indicated antibodies above. UT representative of untreated sample, and H3 serves as a loading control.

3.2 Effects of DNA Damage on HUWE1 and other DDR Proteins

In order to determine potential effects on HUWE1 and DDR proteins in response to DNA damage treatment, protein levels of HUWE1 and associated DNA damage repair proteins were examined under UV and etoposide treatment. A time course experiment for 240 minutes was performed in order to observe changes in DDR proteins. As previously seen, γH2AX protein levels increased in response to DNA damage accumulation in both UV and etoposide treatment conditions. In response to UV treatment, increased levels of HUWE1 were seen initially and then plateaued as the time course continued past 60 minutes (Figure 3.3A). DDR early signaling protein phosphorylated-NBS1 also increased in response to treatment, and similarly protein levels plateaued after 60 minutes. Cells treated with etoposide, showed similar trends, with increased levels of HUWE1, γH2AX and phosphorylated-NBS1 that also plateaued after 60 minutes in response to DNA damage (Figure 3.3B). Therefore, HUWE1 responds to DNA damage signaling in a similar trend as other well-established DDR proteins (γH2AX and phosphorylated-NBS1), suggesting that HUWE1 may be associated in the DDR pathway.

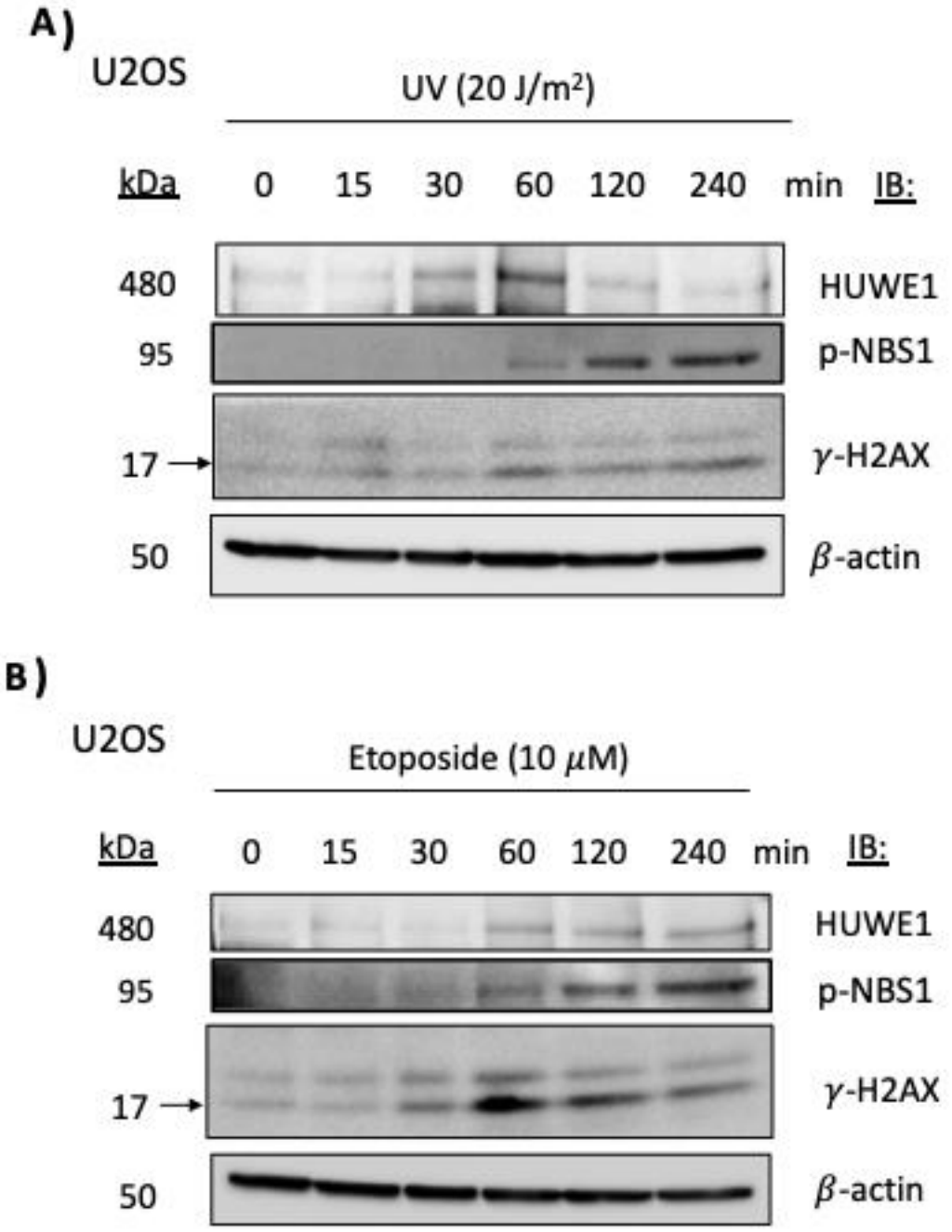


Figure 3.3. Response of HUWE1 and DDR proteins under DNA damage. A) U2OS cells were treated with UV (20 J/m^2) B) U2OS cells were treated with Etoposide (10 μM). Cells were harvested at the various time points over 4 hours and the total cell extract (TCL) was immunoblotted using the antibodies indicated above. DMSO was used as a negative control and β-actin was the loading control.

PARP1 has previously been shown to play a role in regulating the DDR pathway, through PARylation of substrate proteins involved in DDR signaling (d'AMOURS et al. 1999; Haince et al. 2008). In order to measure the response of PARP1 and PAR under DNA damage treatment, a similar experiment was performed as previously described. As shown in Figure 3.4A, in the cells treated with 20 J/m^2 of UV, the level of PARP1 remained relatively stable until the 120-minute time point where it gradually decreased. Cells also demonstrated slightly decreased levels of PAR after 120 minutes following induction of DNA damage. Similar to what was observed with UV treatment, cells treated with 10μM of etoposide, showed decreased levels of PARP1 and PAR after the 60-minute time point (Figure 3.4B). Together, these results revealed that DNA damage induced an increase of HUWE1 and phosphorylated-NBS1 protein levels, however showed an opposing effect on the levels of PARP1 and PAR, which decreased over time following DNA damage.

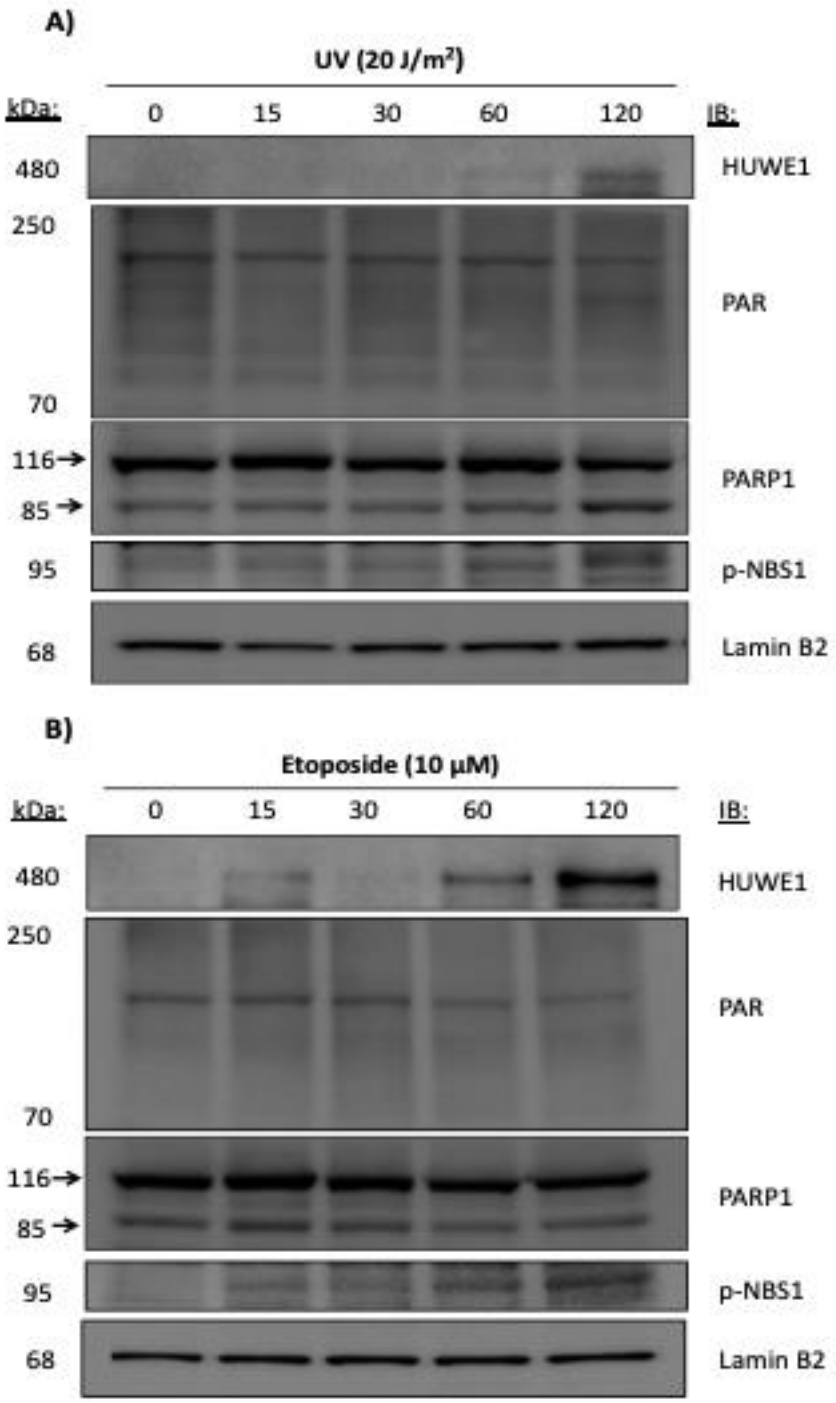


Figure 3.4. Levels of PAR and PARP1 in Response to DNA Damage. A) U2OS cells were treated with 20 J/m² of UV radiation. B) U2OS cells were treated with 10 µM of Etoposide. Cells were treated for 120 minutes and harvested at various time points. PARP1 and PAR protein levels were detected by immunoblotting using antibodies as indicated. Cells were fractionated, and nuclear lysate was used.

3.3 The effect of HUWE1 transient Knockdown on DDR response in U2OS cells

To investigate whether HUWE1 played a role in the DDR pathway, the effect of HUWE1 knocked down in U2OS cells was examined under DNA damage. HUWE1 was transiently knocked down using siHUWE1 or scrambled siRNA for 48 hours, and immunoblot analysis showed that HUWE1 protein levels were partially knocked down, by 38% compared to the control samples (Figure 3.5B). In response to treatment with etoposide (10μM), phosphorylated-ATM and phosphorylated-NBS1 increased as shown prior (Figure 3.5A) in the control samples. However, in siHUWE1 cells, the levels of phosphorylated-ATM and phosphorylated-NBS1 protein levels remained lower by 30% and 59% respectively compared to the siControl cells in response to DNA damage treatment (Figure 3.5C, 3.5D). This result demonstrated that knockdown of HUWE1, influenced phosphorylated-ATM and phosphorylated-NBS1 in response to DNA damage. Since phosphorylated-ATM and phosphorylated-NBS1 are protein markers to demonstrate activation of DDR signaling, the data suggested that HUWE1 played a role in DDR signaling activation.

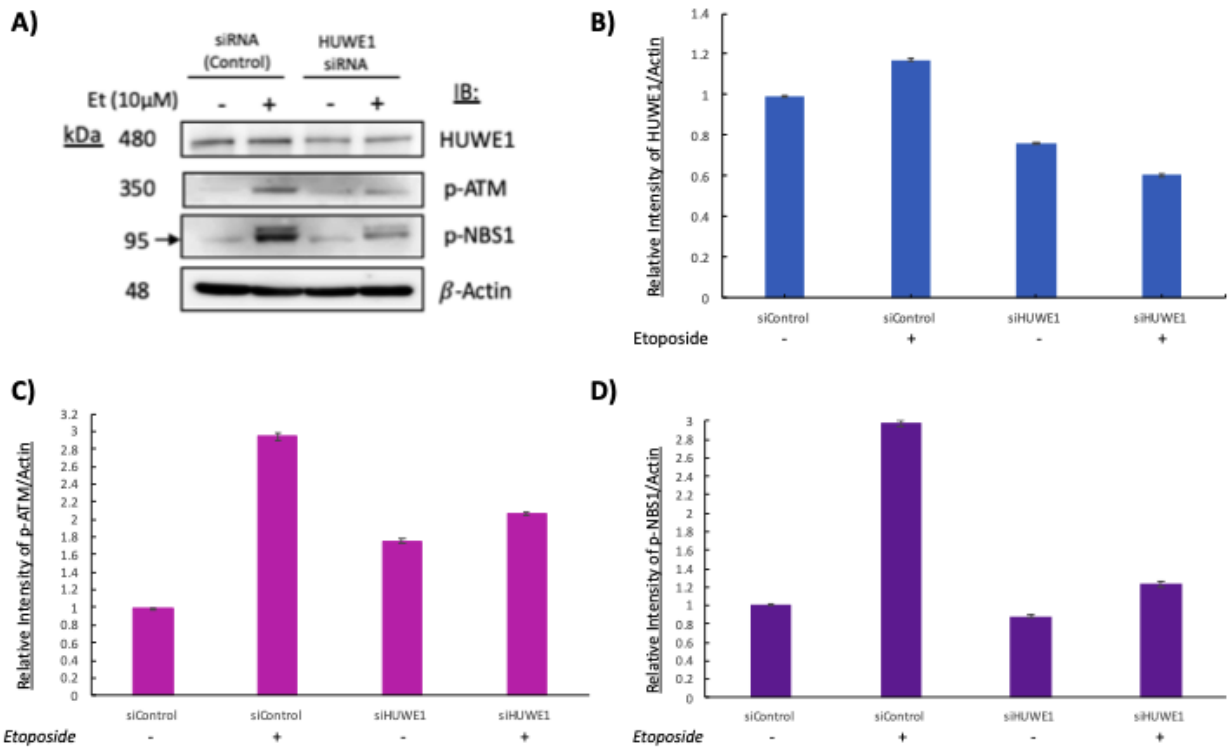


Figure 3.5. Histone and DDR protein response to siRNA Knockdown of HUWE1 in U2OS cells with DNA Damage Treatment. A) U2OS cells were treated for 1 hour with 10 μM of Etoposide and harvested. Total cell lysate (TCL) immunoblotted for HUWE1 and respective DDR proteins. β-Actin used as a loading control. TCL= 20μg of protein loaded. (+) indicates Etoposide treatment and (-) indicated no treatment. B) Quantification of HUWE1 knockdown levels in U2OS cells. Values represents the ratio of HUWE1 to β-Actin for each treatment. C) Quantification of phosphorylated-ATM knockdown levels in U2OS cells. Values represents the ratio of phosphorylated-ATM to β-Actin for each treatment. D) Quantification of phosphorylated-NBS1 knockdown levels in U2OS cells. Values represents the ratio of phosphorylated-NBS1 to β-Actin for each treatment. Quantification densitometry using Fiji, and β-Actin as a control reference.

3.4 Establishment of Stable-Knockdown shHUWE1 U2OS Cell Line

A stable HUWE1 knocked-down U2OS cell line was established in order to show the sustained effect of HUWE1 knockdown on the cellular DNA damage response. A short hairpin RNA (shRNA) lentiviral delivery method was used to knockdown HUWE1 in U2OS cells. shRNA knockdown utilizes a small looped RNA structure that is integrated and continually expressed using a plasmid backbone (pGIPZ). The expressed shRNA can be converted into siRNA in the cell to degrade mRNA in a sequence specific manner to knockdown a target protein. The pGIPZ plasmid contains both a GFP tag for detection of shRNA expression (i.e. Knocked-down HUWE1 cells), and puromycin resistance as a selectable marker in order to propagate only the transduced cells (Figure 3.6A). Two different pGIPZ shHUWE1 constructs, shHUWE1-F6 and shHUWE1-G10, were used in this study to examine the effect of HUWE1 knockdown in U2OS cells. Both pGIPZ shHUWE1 plasmids have been previously validated and used to effectively knockdown HUWE1 in keratinocytes cells (Inoue et al. 2013).

In this study, U2OS shHUWE1-G10 and shHUWE1-F6 cell lines were stably established by transduction using lentiviral approach. Lentiviruses were generated by HEK293 cells transfected with lentiviral packaging plasmids VSVG, PAX2 and the shHUWE1-G10/shHUWE1-F6 plasmids. U2OS shHUWE1-G10 and shHUWE1-F6 cells were selected using puromycin for enriching HUWE1 knockdown cells. The expression of GFP in the shRNA cell lines confirmed the transfection efficiency of the shHUWE1-G10 and shHUWE1-F6 plasmids and their expression in the cells (Figure 3.6B). Immunoblot of HUWE1 further confirmed proper knockdown of HUWE1 protein levels in the shHUWE1-G10 and shHUWE1-F6 cells (Figure 3.6C). Compared to HUWE1 expression in WT U2OS cells, the HUWE1

knockdown efficiency in the shHUWE1-G10 and shHUWE1-F6 cells were 96% and 78% respectively (Figure 3.6C). Thus, the expression of GFP was used as a marker for knockdown of HUWE1 in the cells and was further used in immunofluorescent studies to examine the effect of HUWE1 in the DDR pathway, and the associated proteins in U2OS cells.

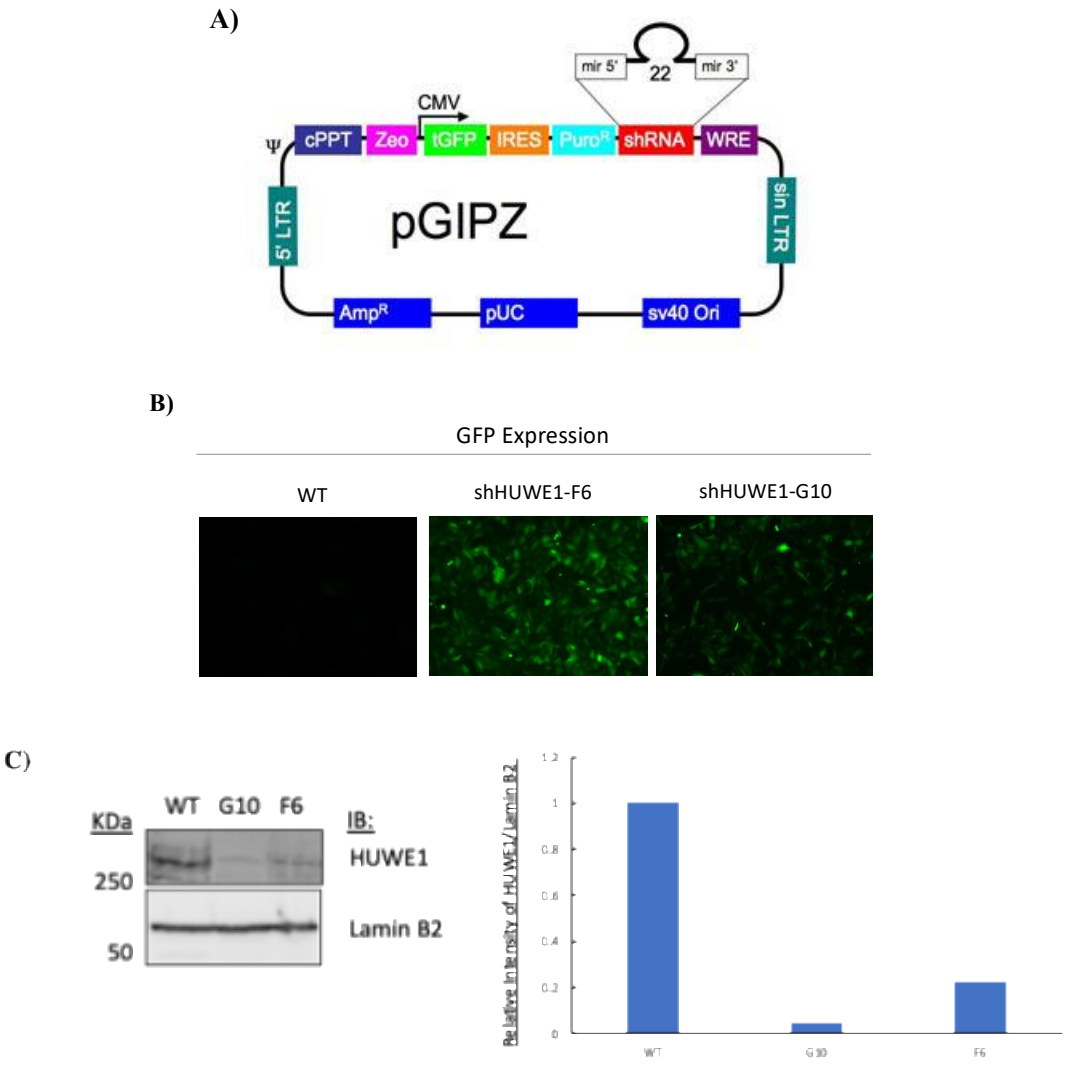


Figure 3.6. Stable knockdown cell lines shHUWE1-G10 and shHUWE1-F6. Cells were stably transfected with lentiviral plasmids shHUWE1-G10 and shHUWE1-F6 respectively. A) pGIPZ shRNA vector expression system used for shHUWE1-G10 and shHUWE1-F6 stably knocked down cell lines ("SPARC BioCentre SickKids ShRNA" n.d.) B) Immunofluorescent imaging of GFP in transfected shHUWE1 cells lines compared to wild type U2OS cells. C) Immunoblot analysis demonstrating knocked down levels of HUWE1 in the shHUWE1-G10 and shHUWE1-F6 cell lines compared to wild-type U2OS cells. Quantification of HUWE1 knockdown levels in U2OS shHUWE1-G10 and shHUWE1-F6 cells. Quantification densitometry using Fiji, and LaminB2 as a control reference. Values represents the ratio of HUWE1 to Lamin B2 for each treatment.

3.5 HUWE1 Inversely Associated with the Levels of PARP1 and PAR in Cells

To understand the role of HUWE1 in the DDR pathway, DNA damage was induced with etoposide and UV in U2OS cells as well as shHUWE1-G10 and shHUWE1-F6 knockdown cells. DNA damage induction was confirmed by increased protein levels of γH2AX in the UV and etoposide treated cells (Figure 3.7). DNA damage increased phosphorylated-ATM, however the knockdown of HUWE1 in the U2OS shHUWE1-G10 and shHUWE1-F6 cells showed an associated decrease of phosphorylated-ATM in response to DNA damage compared to the WT U2OS cells (Figure 3.7). This result supported the association of HUWE1 with phosphorylated-ATM previously shown in the siRNA transient knockdown of HUWE1. Since, phosphorylated-ATM has been shown as an early DDR signaling protein, this evidence suggests that HUWE1 could have a role in the early phase of the DDR pathway. Moreover, overall increased protein levels of PARP1 was seen in the shHUWE1-G10 and shHUWE1-F6 cells compared to the wildtype U2OS cells, regardless of DNA damage treatment (Figure 3.7). This result confirmed the inverse relationship between HUWE1 and PARP1 in U2OS cells.

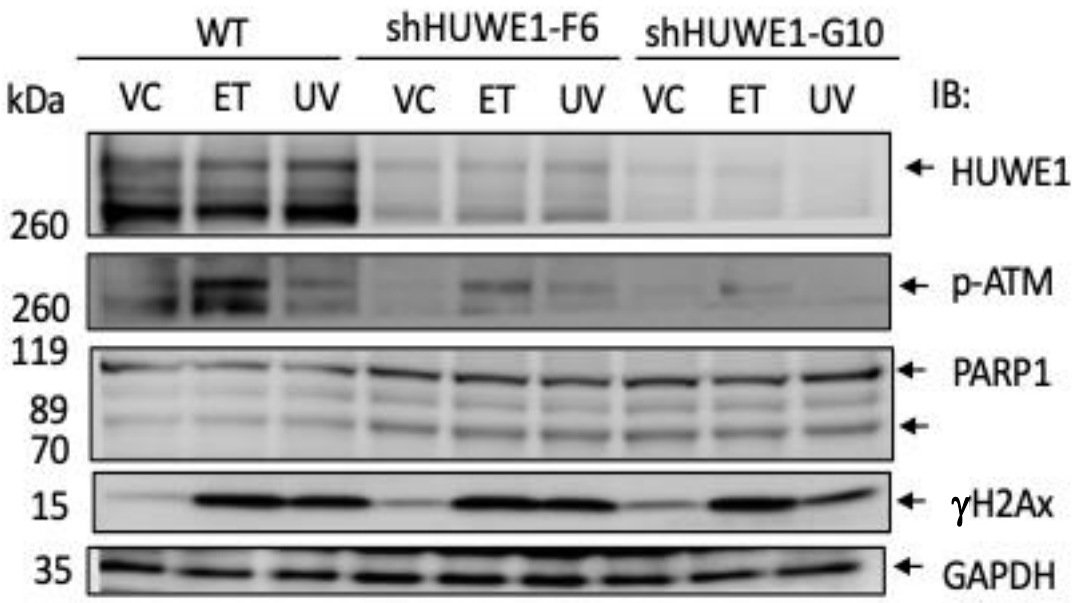


Figure 3.7. DDR response to Knockdown of HUWE1 in shHUWE1-G10 and shHUWE1-F6 U2OS cells treated with Etoposide and UV. Wild-type U2OS and U2OS cells with shHUWE1-G10 and shHUWE1-F6 were treated with 20 J/m^2 of UV radiation or 10μM of Etoposide (topoisomerase II inhibitor) and harvested 1-hour post treatment. Total cell lysate (TCL) was used and immunoblotted with antibodies indicated above.

To further investigate the relationship between HUWE1 and PARP1, immunofluorescence staining of PARP1 was examined in HUWE1 knockdown U2OS cells. GFP intensity in the cells was associated with pGIPZ shHUWE1 plasmid expression (Figure 3.8). As shown in Figure 3.8, the GFP positive shHUWE1-G10 and shHUWE1-F6 cells expressed higher intensity of PARP1 staining globally, compared to the wildtype U2OS cells (Figure 3.8). This data agreed with the previous observation that HUWE1 was inversely associated with the level of PARP1 in U2OS cells, and further demonstrated that HUWE1 may regulate the stability of PARP1.

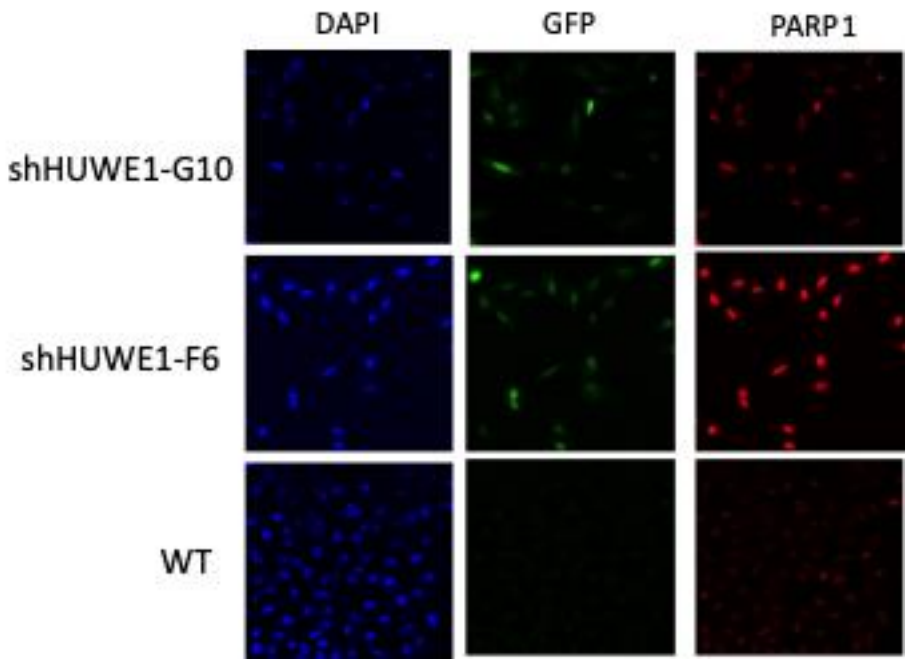


Figure 3.8. Immunofluorescence of HUWE1 vs PARP1 in shHUWE1 U2OS cells. Immunofluorescence staining of Wild-type U2OS and U2OS shHUWE1-G10 and shHUWE1-F6 cells with PARP1 (CST 9542S) antibody. PARP1 visualized with mCherry A555 (Invitrogen A32732) under 20X objective.

Next, the role of HUWE1 in the regulation of PARP1 protein levels and PAR was investigated when HUWE1 was depleted. In order to study the effect of HUWE1 depletion, HUWE1 knockout MEF cells (HUWE1 KO MEF) were used (Hao et al. 2012). The HUWE1 knockout MEF cells were created by a collaborator through the generation of HUWE1 mouse knockout lines using a Cre/*LoxP* recombination strategy (Hao et al. 2012). The MEF cells had deleted HUWE1 at the genomic level, which differed from the previous experiments, in which mRNA degradation based HUWE1 knockdown methods were used and only resulted in partial depletion of HUWE1. HUWE1 WT or KO MEFs were exposed to UV ($20J/m^2$) and immunoblotted for PAR and PARP1 at different time points post-UV treatment (0, 15, 30, 120 mins). In WT MEFs, the levels of PARP1 increased after UV exposure following the similar trend of the DDR marker protein γH2AX. In HUWE1 KO MEFs, global increased levels of PARP1 and PAR were observed compared to HUWE1 WT MEFs (Figure 3.9). Interestingly, in HUWE1 WT MEFs, the levels of PARP1 and PAR increased over time in response to UV treatment, which is opposed to the trend of HUWE1 that showed an early accumulation and a gradual decrease afterwards. However, in HUWE1 KO MEFs, PARP1 and PAR remained at high levels and did not change over the time course in the response to UV exposure (Figure 3.9). Therefore, the depletion of HUWE1 in the KO MEF cells resulted in increased protein levels of PARP1 and PAR, which was consistent with what was demonstrated in previous knockdown experiments, strongly suggesting that HUWE1 negatively regulated the levels of PARP1 and PAR in these cells.

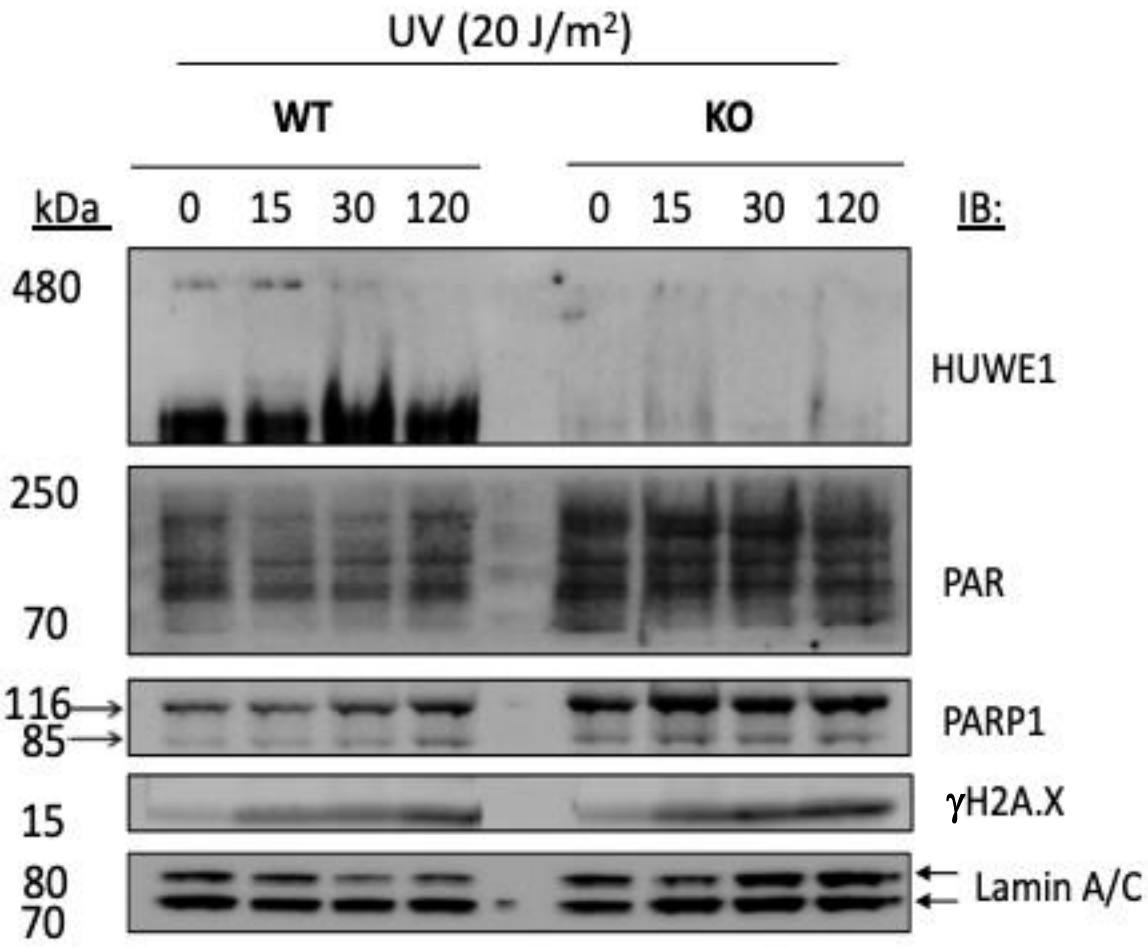


Figure 3.9. PARP and PAR in HUWE1 WT and KO MEF cells with DNA Damage Treatment. Mouse embryonic fibroblast cells (MEF) with wild-type HUWE1 expression and knocked-out HUWE1 (KO) were treated with 20 J/m² of UV radiation over a course of specified time intervals for 2 hours. PARP1 and PAR protein levels were detected by immunoblotting using antibodies as indicated. γH2AX was used as a marker for DNA damage and Lamin A/C was used as a loading control. Cells were fractionated, and nuclear lysate was processed.

3.6 HUWE1 Interacts with PAR and PARP1

PARP1 is known to be recruited in the DDR pathway and regulates substrate PARylation (Satoh and Lindahl 1992). Previous data proposed an inversely associated relationship between the levels of PARP1, PAR and HUWE1, therefore we investigated their interactions in U2OS cells. To determine whether HUWE1 is a PAR-binding protein, co-immunoprecipitation was performed using FLAG-tagged HUWE1 overexpressed 293T cells. The FLAG-tagged HUWE1 overexpressed cells contained both endogenous HUWE1 and the FLAG-tagged construct, whereas the WT control contained only endogenous HUWE1. IgG was used a control for the immunoprecipitation, and a PAR specific antibody readily pulled down HUWE1 in the endogenous and HUWE1 overexpressed cells (Figure 3.10B). However, there was some background PAR seen in the control that was associated with the IgG (Figure 3.10B). This data suggested that HUWE1 is indeed a PAR-binding protein. Moreover, cellular PAR is synthesized by the poly (ADP-ribose) polymerase protein (PARP) members. Since the level of PAR is primarily catalyzed by the enzyme PARP1, coimmunoprecipitation was performed to examine whether HUWE1 could interact with PARP1. Using 293T cells with overexpressed FLAG-tagged HUWE1, the FLAG antibody immunoprecipitation pulled down both FLAG-tagged HUWE1 and endogenous PARP1 in the absence or the presence of DNA damage induced by UV irradiation ($20J/m^2$) (Figure 3.11B). This suggested that HUWE1 could interact with PARP1 in the cell (Figure 3.11B). Collectively, these results showed that HUWE1 forms a complex with both PAR and PARP1.

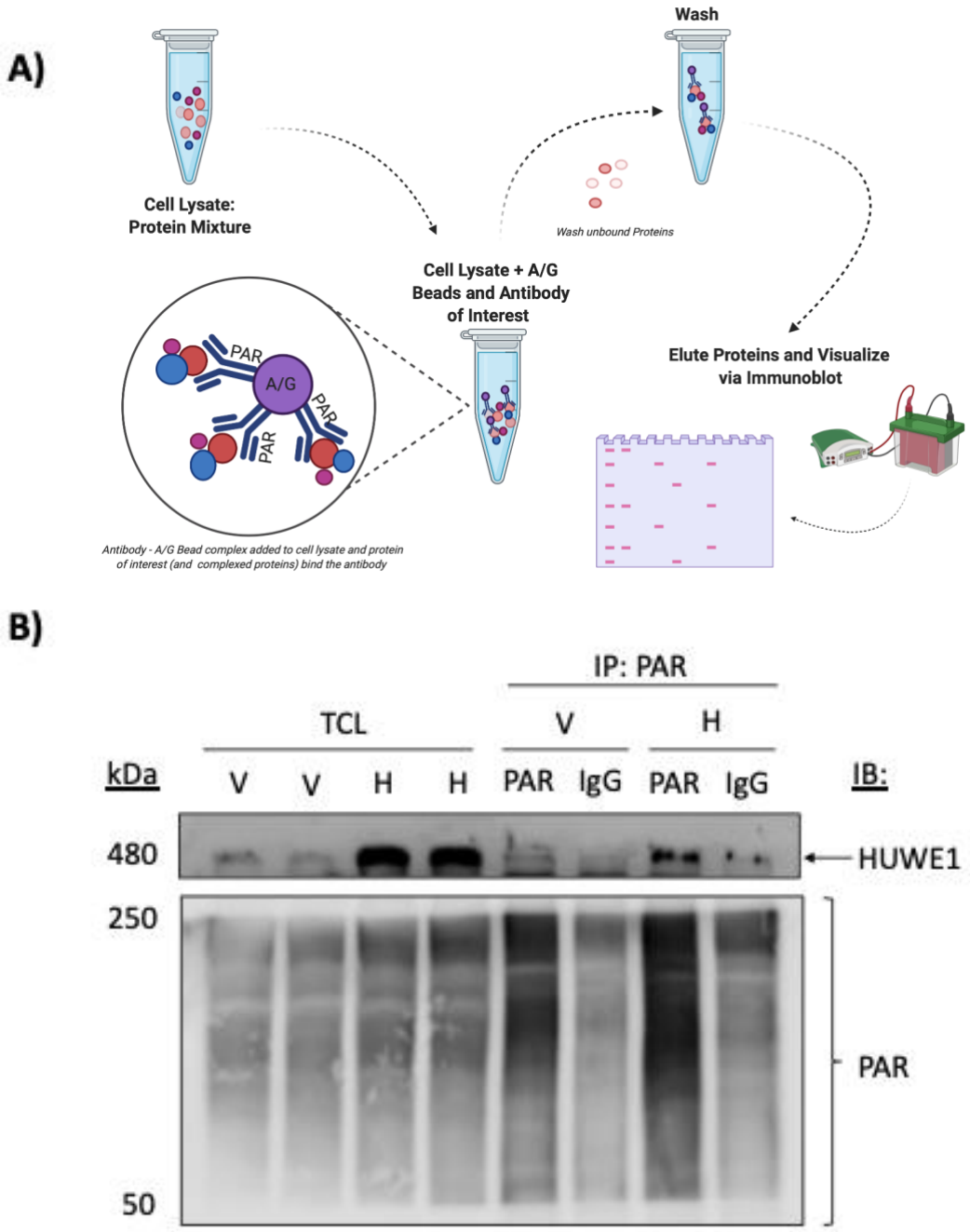


Figure 3.10. HUWE1 interacts with PAR. A) Diagram of immunoprecipitation experimental design (Created with BioRender.com) B) HEK 293T cells were transfected with wild type FLAG-tagged HUWE1 (H) and vector (V) as a control. Cells were lysed and immunoprecipitated with anti-PAR (Mouse). IgG used as a negative control for the immunoprecipitation. TCL represents 10% lysate input, and HUWE1 and PAR were immunoblotted with respective antibodies indicated.

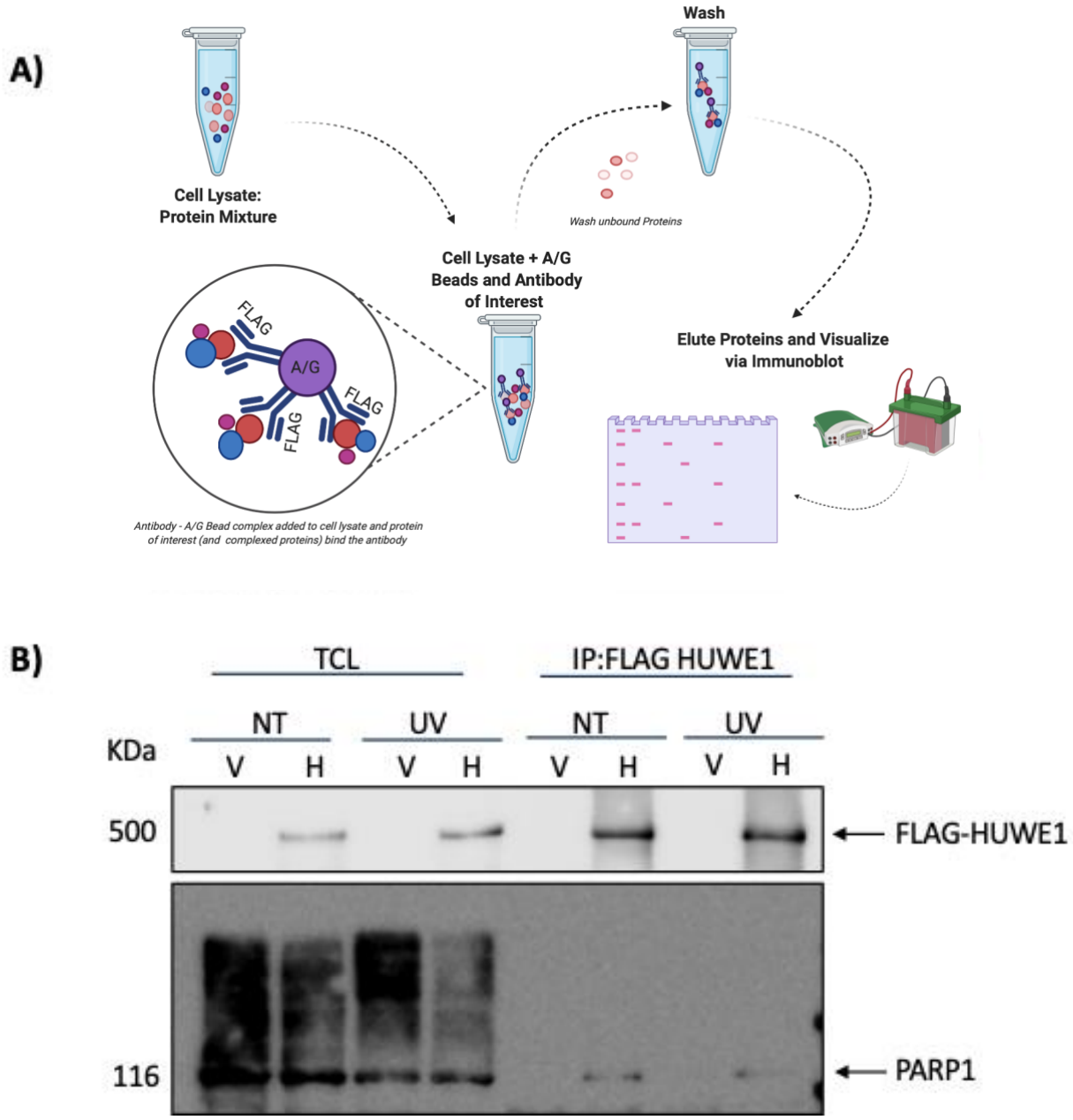
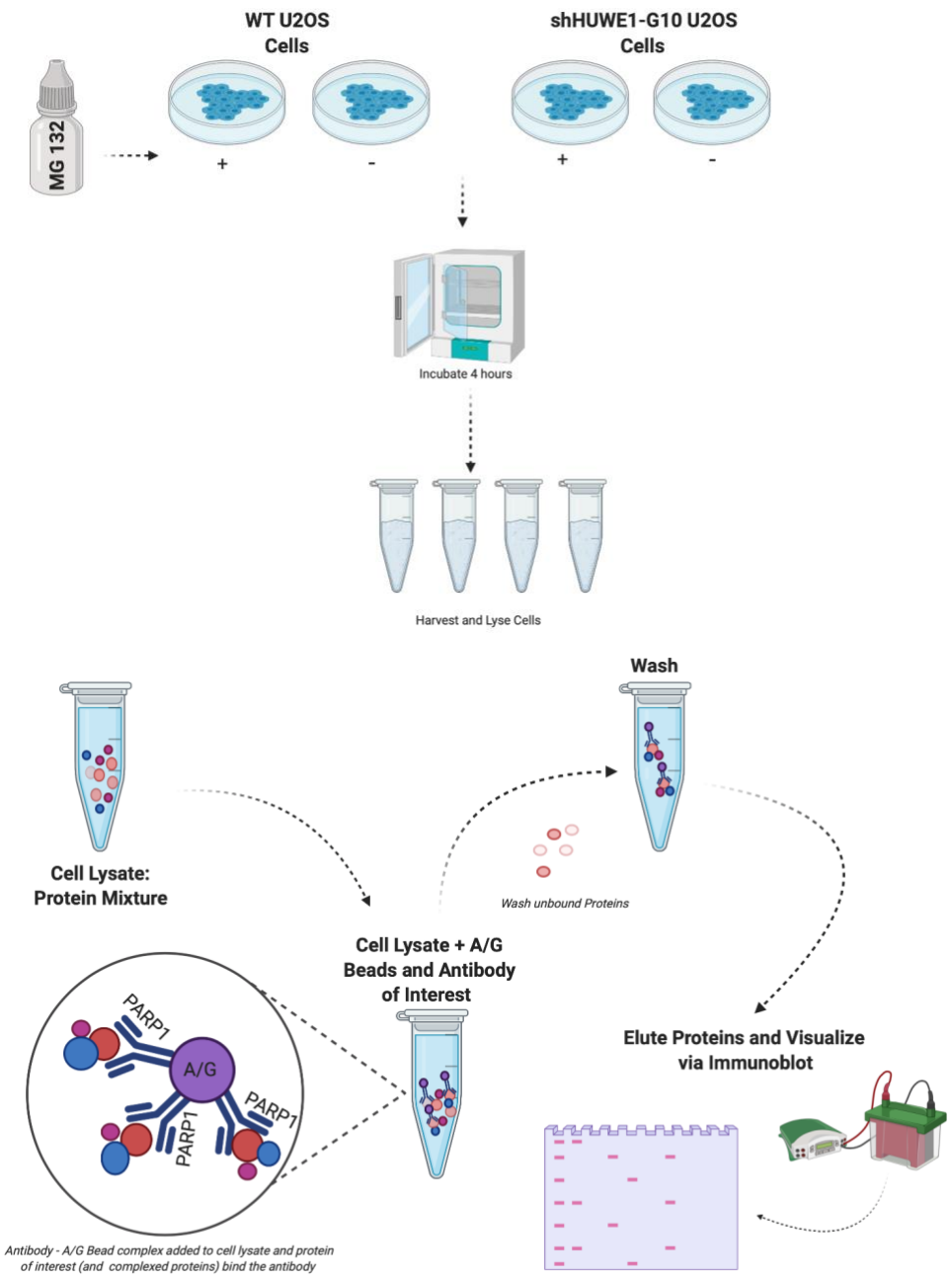


Figure 3.11. HUWE1 interacts with PARP1. A) Diagram of immunoprecipitation experimental design (Created with BioRender.com) B) HEK 293T cells were transfected with wild type FLAG-tagged HUWE1 (H) and vector (V) as a control. Cells were treated with 20 J/m^2 of UV radiation, harvested 1-hour post-treatment and lysed and immunoprecipitated with anti-FLAG. TCL represents lysate input, and NT indicates untreated cells. FLAG and PARP1 were immunoblotted with respective antibodies indicated.

3.7 HUWE1 Mediates PARP1 Ubiquitination

To examine whether HUWE1 inversely regulated PARP1 through ubiquitination, the proteasome inhibitor MG132 was added to the wild type and shHUWE1-G10 knockdown U2OS cells. PARP1 ubiquitination was detected by immunoblotting with ubiquitin antibody following immunoprecipitation with PARP1 antibody (Figure 3.12A). As seen in Figure 3.12B, wild type U2OS cells treated with MG132 had higher levels of ubiquitinated PARP1 compared to the cells with knocked-down HUWE1 protein levels. Since, decreased HUWE1 protein levels impeded PARP1 ubiquitination, this data demonstrated that HUWE1 contributes to the destabilization of PARP1 through PARP1 ubiquitination in the cell.

A)


WT U2OS Cells
shHUWE1-G10 U2OS Cells
MG 132
+
-
+
-
Incubate 4 hours
Harvest and Lyse Cells
Wash
Cell Lysate: Protein Mixture
Wash unbound Proteins
Cell Lysate + A/G Beads and Antibody of Interest
PARP1
A/G
PARP1
PARP1
Elute Proteins and Visualize via Immunoblot
Antibody - A/G Bead complex added to cell lysate and protein of interest (and complexed proteins) bind the antibody

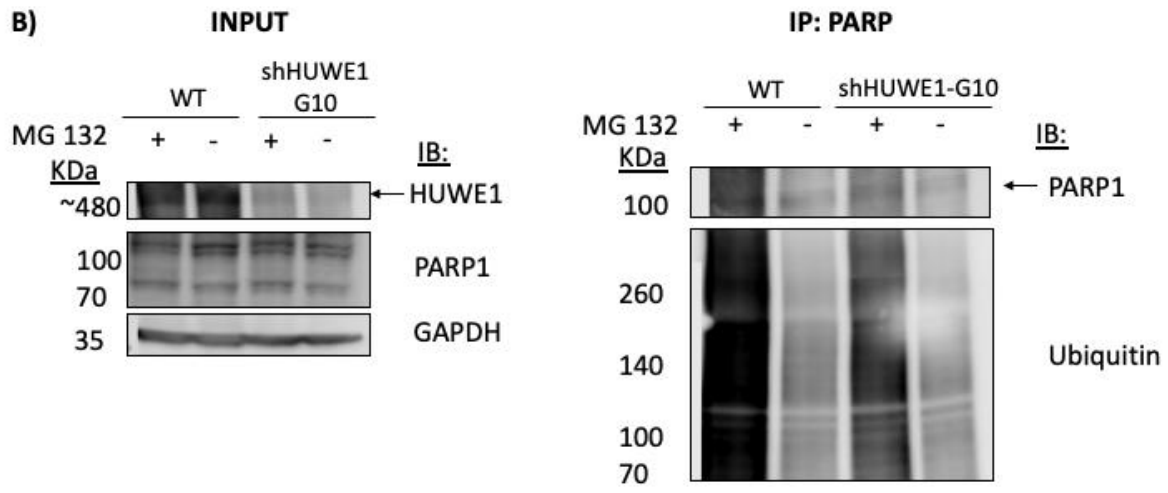


Figure 3.12. HUWE1 Regulates Stability of PARP1 through Ubiquitination. A) Diagram of immunoprecipitation experimental design (Created with BioRender.com) B) Wild-type U2OS and U2OS cells with knocked down HUWE1 (shHUWE1-G10) were treated with MG132 (Proteasome Inhibitor) for 4 hours. In order to determine whether the HUWE1 regulated the ubiquitylation of PARP1, a PARP1 immunoprecipitation using protein A/G beads was preformed, and samples were immunoblotted for ubiquitin.

Chapter 4: Discussion and Future Directions

In order to maintain genome integrity, eukaryotic cells have evolved a complex signalling response through the DDR pathway to promote proper DNA repair. The regulation of the DDR pathway in response to DNA damage is complex and involves coordination of various proteins which could be promoted by different types of post-translational modifications used in cell signalling. However, due to the complexity of the DDR, the mechanism by which the pathways are regulated are not well understood. HUWE1 is an E3 ligase that has been implicated to be involved DNA damage repair. Apart from HUWE1 ubiquitination mediated signalling, HUWE1 also contains other domains including the WWE domain that binds ADP-ribose. ADP-ribose is a subunit of PAR chains that mediate PARylation signalling (Wei and Yu 2016). PARylation is also a post translational modification associated in the DDR pathway, but the mechanism of how the signalling is mediated in the DDR, is not well characterized. Moreover, the functional association between HUWE1 and PARylation proteins including PARP1 has not been reported previously. Therefore, the purpose of this study was to elucidate how HUWE1 impacts the DDR pathway and the mechanism by which HUWE1 regulates associated DNA damage repair proteins including PARP1.

4.1.HUWE1 is Involved in the DDR Pathway

The first aim of the study was to understand how HUWE1 is involved in the DDR pathway and its association with various DDR proteins. In order to measure the DDR, DNA damage conditions were surveyed in order to optimize sufficient DNA damage activation with the desired DNA damage treatments. UV radiation and etoposide treatment were utilized to induce DNA breaks in order to survey proteins associated in DDR pathways. DSB could induce the most dramatic and detrimental DNA damage effects in cells, and improper repair mechanisms in

those pathways can result in carcinogenesis or cell death (Ciccia and Elledge 2010). HUWE1 has been previously studied under DNA damage conditions to induce SSB and DSB, therefore the effects on associated proteins were measured using similar DNA damage agents such as etoposide and UV treatment (Ciccia and Elledge 2010; Kao, Wu, and Wu 2018). γH2AX, a well-established marker for DNA damage, was surveyed in multiple cell lines, and time points. Time points as early as 30mins-1hour were shown to elicit γH2AX accumulation in response to DNA damage induced by UV ($20J/m^2$) and etoposide (10μM) treatment (Figure 3.1, 3.2). Therefore, γH2AX was used as a DNA damage marker for the early DDR in this study.

In order to understand the role of HUWE1 in the DDR pathway under DNA damage treatment, U2OS cells were treated with both UV and etoposide. DNA damage treatment increased protein levels of HUWE1, and the pattern followed the activation of DDR proteins including γH2AX and phosphorylated-NBS1 as early as 30 minutes in U2OS cells (Figure 3.3). U2OS cells are cancerous and have intrinsic genome instability and basal level of DDR. The addition of DNA damage agents (UV and etoposide) caused remarkably elevated DDR as shown by the increased activation of DDR proteins. ATM is an important kinase that when activated, phosphorylates downstream DDR proteins including NBS1 (Lavin et al. 2015). The two proteins interact in order to further propagate DNA repair protein recruitment to the DNA lesion and mediate DSB response (Ciccia and Elledge 2010; Lee et al. 2003, 2013; R. S. Williams et al. 2009). Therefore, phosphorylated-ATM and phosphorylated-NBS1 are well established DDR signalling proteins, and the increase in activation of these proteins in response to DNA damage has been previously shown and confirmed in our study (Ciccia and Elledge 2010; Lavin et al. 2015; Lee et al. 2003). Interestingly, the increase of HUWE1 protein levels in response to DNA

damage follows the increased activation of DDR proteins exemplified by phosphorylated-NBS1. Therefore, the experiment showed that HUWE1 protein levels increase in response to DNA damage, similarly to the early response DDR protein.

Since increased phosphorylated-NBS1 in correlation to HUWE1 levels were observed, we hypothesized that HUWE1 may influence phosphorylated-ATM and phosphorylated-NBS1 in a non-proteolytic manner in the DDR pathway. This was tested by inducing DNA damage in HUWE1 knockdown cells and examining the effect of decreased HUWE1 levels on phosphorylated-ATM and phosphorylated-NBS1. The data supported that when HUWE1 was knocked down, levels of phosphorylated-ATM and phosphorylated-NBS1 decreased respectively (Figure 3.5, Figure 3.7). This data showed the non-proteolytic association between HUWE1 protein levels in U2OS cells and phosphorylation of NBS1 and ATM. Interestingly, a previous study indicated a relationship between HUWE1 and phosphorylated-ATM, where HUWE1 deficient MEF cells elicited decreased levels of phosphorylated-ATM in response to genotoxic stress (Hao et al. 2012). The results from this study showed a similar relationship between HUWE1 and phosphorylated-ATM in U2OS cells under DNA damage. Moreover, the results showed the potential correlation between HUWE1 and phosphorylated-NBS1 under DNA damage, which has not been well studied or previously shown.

Since ATM and NBS1 are involved in early DDR signalling recruitment, the subsequent decreased activation of both proteins when HUWE1 was knocked down provided evidence that HUWE1 may play a role in the regulation of early activation of DDR proteins in DSB repair. However, further studies would be necessary to understand the mechanism by which HUWE1

regulates the activation of ATM and NBS1 in early DDR signalling. Thus, it would be beneficial to do an immunoprecipitation to determine if HUWE1, phosphorylated-NBS1 and phosphorylated-ATM proteins are potentially interacting in the same complex. Moreover, in order to further understand the role of HUWE1 on DDR protein recruitment it would be important to visualize protein recruitment (DNA damage foci) in response to DNA damage through immunofluorescence studies. Therefore, cells with endogenous HUWE1 and knocked down HUWE1 levels can determine if HUWE1 influences localization or recruitment of various DDR proteins including ATM, NBS1 and downstream proteins including 53BP1 and BRCA1.

4.2. HUWE1 Interacts with PAR and PARP1

PARP1 catalyzes the PARylation (addition of PAR) of substrates, as another form of post-translational modification, which serves as a unique signal to mediate DDR protein recruitment (d'AMOURS et al. 1999; Kim, Zhang, and Kraus 2005; Ray Chaudhuri and Nussenzweig 2017). PARylation signalling is recognized by PAR binding to specific structural PAR recognition motifs on various proteins (Ray Chaudhuri and Nussenzweig 2017; Wei and Yu 2016). There are many established PAR binding sites including WWE domains. However, the regulation of PARP1 and PARylation signalling in the DDR is not well characterized or understood. Although HUWE1 contains a WWE domain, the regulation of PARP1 and PAR binding has never been previously shown. Recent studies by a collaborative group determined *in vitro* binding of ADP-ribose (subunit of PAR chains) to the HUWE1 WWE domain (*data not published*). Moreover, immunoblot analysis of HUWE1 protein levels in response to DNA damage showed an inverse relationship between HUWE1 with PAR and PARP1 levels in U2OS cells (Figure 3.4).

Therefore, this study characterized the interaction of HUWE1 with PAR and PARP1, in order to examine whether they are associated functionally in the DDR pathway.

An immunoprecipitation using FLAG-tagged HUWE1 overexpressed 293T cells was done in order to determine if FLAG-tagged HUWE1 interacted with PARP1. It was confirmed that the FLAG-tagged HUWE1 was able to interact with PARP1 regardless of DNA damage treatment (Figure 3.11). Moreover, another student in the lab using a reciprocal immunoprecipitation, showed that PARP1 was also able to pull down HUWE1 (*Data not shown*). Since the results were validated using reciprocal immunoprecipitations, it strongly suggests that HUWE1 forms a complex with PARP1 in cells. However, in order to validate if HUWE1 directly binds to PARP1, an *in vitro* pull-down assay using recombinant PARP1 and HUWE1 could be performed.

Furthermore, since HUWE1 was shown to interact with PARP1, the next study aimed to characterize if the interaction with HUWE1 was PAR dependant. An immunoprecipitation assay with PAR antibody was able to pull down both ectopically expressed FLAG-HUWE1 and endogenous HUWE1 (Figure 3.10), supporting the interaction of HUWE1 with PAR. However, further experiments are needed in order to determine if the interaction is WWE domain dependant. The collaborator determined that the mutated residues (Y1658A, W1619A, Q1667A, and R1676A) in the HUWE1 WWE domain disrupted ADP-ribose binding using a biochemical assay (*Data not shown*). Therefore, a biochemical binding assay using recombinant HUWE1 WWE domain, and mutated (Y1658A, W1619A, Q1667A, and R1676A) HUWE1 WWE domains, could be used to further validate if HUWE1 directly interacts with PAR through the WWE domain.

4.3. HUWE1 Regulates PAR and PARP1 Stability

Moreover, since HUWE1 immunoprecipitated with both PAR and PARP1, it would be important to understand the functional outcome of the interaction. The results showed that in response to DNA damage treatment HUWE1 levels increased while PAR and PARP1 levels decreased in wild type U2OS cells (Figure 3.4). This inverse relationship was further supported through both immunofluorescence and immunoblot analysis, with increased levels of PARP1 in U2OS HUWE1 knockdown cells (Figure 3.7 and Figure 3.8). Furthermore, MEF cells with knocked out HUWE1 protein levels similarly showed overall PAR and PARP1 level increase regardless of DNA damage treatment (Figure 3.9). Therefore, HUWE1 negatively regulated with PAR and PARP1. Noticeably, HUWE1 regulated the stability of both PAR and PARP1 regardless of DNA damage treatment.

Although there was a general inverse relationship between the levels of HUWE1 and the levels of PARP1 and PAR, PARP1 response to DNA damage differs greatly in different cell lines. U2OS cells showed a slight increase of PARP1 and a subsequent decrease through the time course (Figure 3.4), indicating U2OS cells recruit and turnover PARP1 very quickly in response to DNA damage (Caron et al. 2019). However wild type MEF cells show gradually increased levels of PARP1 in response to DNA damage (Figure 3.9). Therefore, further time course studies need to be investigated with different types of DNA damage agents to determine how the recruitment and stability of PARP1 in response to DNA damage is regulated in various cell lines.

4.4. HUWE1 Mediates PARP1 Ubiquitination

HUWE1 is an E3 ligase and known to regulate the stability of various proteins involved in DDR through ubiquitylation. Therefore, the last aim of the study was to examine whether HUWE1 could ubiquitylate PARP1. A cellular ubiquitination assay was performed to determine whether HUWE1 mediated PARP1 ubiquitination with MG132 treatment. The data demonstrated that HUWE1 is a PARP1 E3 ligase, because ubiquitination was compromised in cells with knocked down HUWE1 levels (Figure 3.12). This suggests that HUWE1 is able to regulate the stability of PARP1 through ubiquitination. However, in the cell there are various E3 ligases known to regulate PARP1 including RNF 146, and RNF 144 (Kang et al. 2011; Ye Zhang et al. 2017). Therefore, further experiments are needed to determine the distinct role of HUWE1 in the regulation of PARP1 by ubiquitylation.

In order to further validate the mechanism by which HUWE1 regulates PARP1 stability, a biochemical ubiquitination assay could be performed using recombinant PARP and HUWE1 proteins. Moreover, in order to further validate if HUWE1 is an E3 ligase for PARP1, a ubiquitination assay using a HUWE1 HECT domain dead mutant (inhibited ubiquitylation activity) could be performed. Lastly, to determine whether PARP1 activity is required for HUWE1 mediated PARP1 ubiquitination, a PARylation inhibitor (PJ34) could be used in the ubiquitination assay to prevent PARP1 auto PARylation. Moreover, WWE domain mutants could be used to further confirm whether the E3 ligase activity of HUWE1 is PAR dependant in the ubiquitination assay. Therefore, these experiments could provide further insight into the mechanism of PARP1 regulation by HUWE1.

4.5. Limitations and Implications of the Current Study

This research demonstrated that HUWE1 plays a role in the DDR pathway and is associated with DNA repair proteins including phosphorylated-ATM, phosphorylated-NBS1, and PARP1. The results showed that HUWE1 influenced the regulation of activated ATM and NBS1, in response to DNA damage. Moreover, this study also demonstrated a novel interaction between HUWE1 with PARP1 and PAR in cells and provided evidence that HUWE1 could be a novel E3 ligase that regulates PARP1 stability.

Although the results provided new insight to HUWE1's role in the DNA damage repair pathway the study has various limitations. Firstly, since HUWE1 is a large protein and well known for its E3 ligase function, the mechanism of HUWE1's signaling role and association to phosphorylated-NBS1, phosphorylated-ATM needs to be further established. Moreover, although HUWE1 interacts with both PAR and PARP1, surprisingly our study showed that this interaction is independent of DNA damage. This data is unexpected because PARP1 has been widely associated in the DNA repair pathway and can directly bind damaged DNA sites (Ray Chaudhuri and Nussenzweig 2017; Wei and Yu 2016). Thus, a limitation to our study is that the role of HUWE1 and PARP1 regulation in the DDR pathways remains unclear. Therefore, more studies are required to elucidate the regulation of PARP1 by HUWE1 and its association with DDR. It is well known that PARP1 regulates DDR signalling proteins including ATM, NBS1, MRE11 and downstream DDR proteins including BRCA1 (Haince et al. 2007, 2008; M. Li et al. 2013; M. Li and Yu 2013; Murcia et al. 2001). However, PARP1 is regulated by multiple E3 ligases including RNF146 and RNF144, therefore the biological importance of the interaction between HUWE1 and PARP1 in the DDR pathway also still needs to be further characterized

(Kang et al. 2011; Ye Zhang et al. 2017). In order to better characterize the role of HUWE1 and its relationship with PARP1, the creation of CRISPR HUWE1 knockout cells would be beneficial to better understand the full effect of HUWE1 contribution to the pathway. Moreover, experiments testing simultaneous deletion of multiple DDR proteins in association with HUWE1, and reconstitution of those proteins in relation to HUWE1 may also provide more insight *in vivo* of how HUWE1 regulates proteins in the DDR pathway in future studies.

In conclusion, the stringent regulation of DNA damage in cells is very important in maintaining genomic integrity. DNA lesions that are regulated through error-prone DNA repair pathways, or not properly repaired, promote an increased rate of mutagenesis. This is associated with a higher risk of carcinogenesis or associated cell death. Therefore, the implications of this study are important because it provides a better understanding of HUWE1's role in the DDR pathway. Further understanding of the mechanisms that regulate the DDR pathway are important to create therapeutics targeting the aberrant DDR pathways. The use of PARP1 inhibitors as a cancer treatment has become an effective therapeutic that utilizes the DNA repair pathway to selectively induce cancer cell death. Therefore, this study helped to better characterize the regulation of PARP1 by HUWE1 and could potentially help provide insight to guide effective use of PARP1 inhibitor therapeutics in DNA repair deficient cancers. Lastly, this study also helped to further improve and understand the role of HUWE1 in the DDR pathway and its novel interaction with PARP1 and PAR.

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Appendix

Supplementary Data

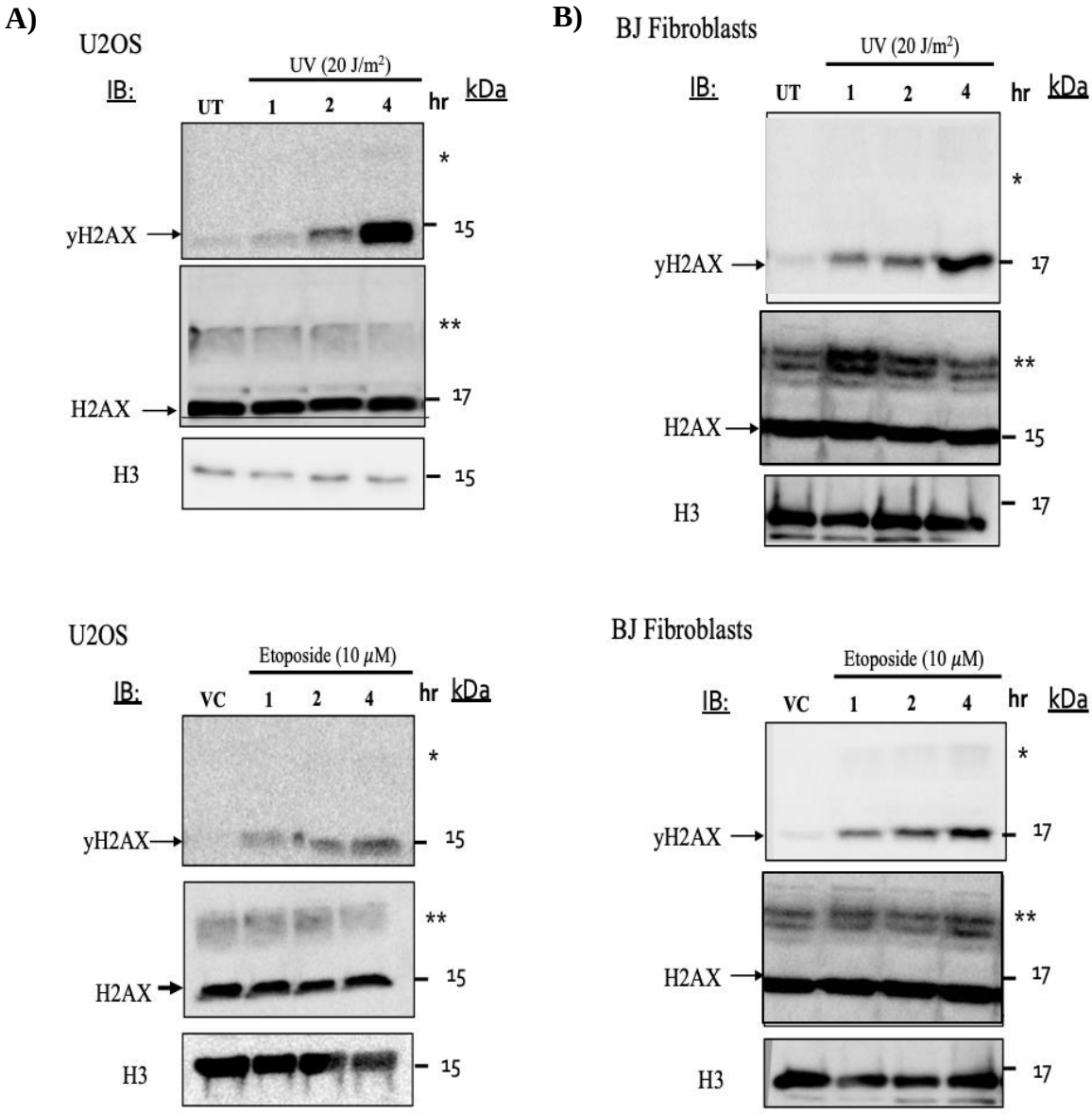

Figure S1. Histone Response to DNA Damage: Time-Course Experiment. Histone activation and various modifications in A) U2OS, and B) BJ Fibroblasts cells under induced DNA damage. Cells were treated with Etoposide (10μM) and UV radiation (20J/m^2) treatment for 30min-4 hours. Immunoblot visualization using 20μg of nuclear extract with indicated antibodies above. UT representative of untreated sample, VC representative of vehicle control (DMSO) and H3 serves as a loading control.