

The Pennsylvania State University

The Graduate School

College of Medicine

**HUWE1 INTERACTS WITH PCNA TO ALLEVIATE
REPLICATION STRESS**

A Dissertation in

Biomedical Sciences

by

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Submitted in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

December 2016

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Abstract

The integrity of the genome relies on the accurate and faithful duplication of genetic information from a parental cell to its progeny during each cellular division. Defects in DNA replication, DNA damage response, or DNA repair compromise genomic stability and promote cancer development as well as other diseases. In particular, unrepaired DNA lesions can arrest the progression of the DNA replication machinery during S-phase, causing replication stress, mutations, and DNA breaks. HUWE1 is a HECT-type ubiquitin ligase that targets proteins involved in cell fate, survival and differentiation. Here, we report that HUWE1 is essential for genomic stability, by promoting replication of damaged DNA. We show that HUWE1-knockout cells are unable to mitigate replication stress, resulting in replication defects and DNA breakage. Importantly, we find that this novel role of HUWE1 requires its interaction with the replication factor PCNA, a master regulator of replication fork restart, at stalled replication forks. Finally, we provide evidence that HUWE1 monoubiquitinates H2AX to promote signaling at stalled forks. Altogether, our work identifies HUWE1 as a novel regulator of the replication stress response.

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List of Abbreviations

APIM	AlkB Homologue 2 PCNA-Interacting Motif
ARF-BP1	ARF-Binding Protein 1
ARLD	Armadillo Repeat-Like Domain
ATM	Ataxia Telangiectasia Mutated
ATR	Ataxia Telangiectasia and Rad3 Related
BER	Base Excision Repair
BH3	Bcl-2 Homology 3 Domain
BrdU	Bromodeoxyuridine
CDC6	Cell Division Cycle 6
CDK2	Cyclin Dependent Kinase 2
Cdt1	Chromatin Licensing and DNA Replication Factor 1
CFS	Common Fragile Site
CldU	5-Chloro-2'-Deoxyuridine
DDR	DNA Damage Response
DDT	DNA Damage Tolerance
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
DSB	Double Strand Break
EdU	5'-Ethynyl-2'-Deoxyuridine
GST	Glutathione S-Transferase
HDR	Homology-Directed Repair
HECT	Homologous to E6-AP Carboxyl Terminus
HectH9	Homologous to E6-AP Carboxyl Terminus Homologous Protein 9
HR	Homologous Recombination
HU	Hydroxyurea
HUWE1	HECT, UBA, and WWE Domain-Containing Protein 1
ICL	Interstrand Crosslink Repair
IDCL	Interdomain Connecting Loop
IdU	5-Iodo-2'-Deoxyuridine
iPOND	Isolation of Proteins on Nascent DNA
Lasu1	Large Structure of UREB1
MCM	Minichromosome Maintenance Complex
MMR	Mismatch Repair
MULE	Mcl-1 Ubiquitin Ligase E3
NER	Nucleotide Excision Repair
NLS	Nuclear Localization Signal
ORC	Origin Recognition Complex
PCNA	Proliferating Cell Nuclear Antigen
PI	Propidium Iodide
PIP	PCNA-Interacting Peptide
Pol	Polymerase
Pre-RC	Pre-Recognition Complex
PRR	Post-Replicative Repair
RBR	Ring Between Ring
RFC	Replication Factor C

RING	Really Interesting New Gene
RNA-Seq	Ribonucleic Acid-Sequencing
rNTP	Ribonucleotide Triphosphate
RPA	Replication Protein A
SCR	Sister Chromatid Recombination
ssDNA	Single-Stranded DNA
TLS	Translesion Synthesis
UBA	Ubiquitin-Associated Domain
UV	Ultraviolet
WWE	Protein-Protein Interaction

Acknowledgements

I would first like to thank my thesis advisor, Dr. George-Lucian Moldovan, for being an incredibly wonderful mentor and for his dedicated investment in my scientific development. I am very fortunate to have been under his guidance and have direct exposure to his wealth of knowledge, work ethic, and commitment to science. The past few years as a student in his lab have been the most transformative years for me personally, and as a budding scientist. Thank you for being the best mentor I could have asked for, for always being on my side, and for always challenging me to grow as a scientist.

I would also like to extend my sincerest gratitude to the committee members for their continuous support and commitment throughout my graduate school career. Thank you, Dr. Cristina Truica, for your words of encouragement, time, and commitment as a member of the committee. Thank you, Dr. Broach, for your guidance and your invaluable intellectual input to my thesis work. Thank you, Dr. Vrana, for being a wonderful source of support and advice from very early on in my graduate studies, and for encouraging my scientific career development. Thank you, Dr. Ralph Keil, for being a pivotal figure during my time here. I express my gratitude for all of your guidance, unwavering support, and for helping me overcome difficult times throughout my graduate career. Thank you for always having your office door open (when you were there).

Next, I'd like to thank the past and present lab members of the Moldovan lab, for providing a wonderful and friendly work atmosphere. Specifically, I would like to thank Dr. Claudia Nicolae for all of her help with experiments and troubleshooting, and for being so welcoming and warm in the lab. Thank you, Claudia, for creating a jovial work environment for the rest of the team, and for all of the work you do for the lab. I would like to thank the rest of the lab members for giving me another reason to look forward to coming into the lab every day. I am incredibly fortunate to be able to share memories with them from both inside and outside of the lab.

I would like to thank Sanziana Rotariu, Hejuan He, Daniel Constantin, and Dr. Rocio Delgado for contributions to this work, and Dr. Alan D'Andrea, Dr. Stefan Jentsch, Dr. Wafik El-Deiry, Dr. David Cortez, Dr. Wojciech Piwko, Dr. Kyungjae Myung, Dr. David Kozono, Dr. Sergei Grigoryev, Dr. Kristin Eckert, Dr. Thomas Spratt, Dr. Faoud Ishmael, Dr. Laura Carrel, and Dr. Gregory Yochum for materials, support, and advice. I would like to thank Dr. Yuka Imamura for her technical and logistical assistance with the RNA-sequencing. I would like to thank our collaborators, Dr. Veronique Smits and Dr. Raimundo Freire, for providing the co-immunofluorescence protocol and their contributions to the immunofluorescence experiments. In addition, I would like to thank the current students of the Broach lab for scientific discussions and for the friendships that were created.

Finally, I would like to acknowledge my wonderful parents and brother, and thank them for always believing in me and encouraging me to evolve professionally and personally. I am forever indebted to my parents' endless self-sacrifice, bottomless love, and never-ending support. They are the strongest and most selfless individuals I know and I would not have come this far without them. And thanks to my older brother, Dustin, because I admire his innate thirst for knowledge, wisdom, wit, and resilience. I am fortunate to have his support, guidance, and existence in my life.

Dedication

This is dedicated to my parents, with more love and gratitude than can ever fit these pages. Thank you for giving me life, and for giving my life meaning.

Chapter 1. INTRODUCTION

1.1. GENOMIC STABILITY AND REPLICATION STRESS

Genome instability is a hallmark of cancer and also results in cellular aging and other diseases (Aguilera and García-Muse, 2013). Thus, maintaining the integrity of the genome is critical. Genomic stability is dependent on the accurate duplication and faithful transmission of genetic information from a parental cell to its progeny during each cell division event. The cell is most vulnerable to genomic instability during DNA replication; therefore, the initiation and completion of accurately copying billions of nucleotides must be tightly regulated to ensure fidelity and avoid catastrophe. This introduction will cover the process of DNA replication, the definition, sources, and outcomes of replication stress, as well as review the known mechanisms of how the cell addresses replication stress to avoid the onset of genomic instability. Furthermore, this chapter will introduce two players involved in the replication stress response: PCNA, a well-known player involved in responding to replication stress, as well as a novel protein involved in the replication stress response known as HUWE1.

1.1.1. DNA Replication

DNA replication is a highly dynamic and complex process that involves the accurate duplication of billions of nucleotides in mammalian cells. This process is divided into two defined steps, with each step taking place during a distinct phase of the cell cycle. Starting in the G1 phase of the cell cycle, the cell prepares for DNA replication through a process known as origin licensing. This step prepares the chromatin for replication and ensures that replication only takes place once per cell division by the assembly of the pre-recognition complex (pre-RC) (Masai et al., 2010). During origin licensing, the six subunit ATPase called the origin recognition complex (ORC) is

recruited and binds to discrete replication origins across the genome. This is followed by the subsequent binding of the hexameric MCM2-7 complexes, ATPase CDC6, and licensing cofactor CDT1. Altogether, these proteins form the pre-RC. Once the pre-RC is formed, the origin is licensed, CDC6 and CDT1 are released, and ORC and MCM2-7 are retained at these origins for DNA replication (Boos et al., 2012).

Following origin licensing, the origin fires and DNA replication occurs during S-phase. This is triggered by cyclin-dependent kinase 2 (CDK2) activating MCM2-7 to allow for replisome loading onto DNA (Boos et al., 2012). CDC45 and the tetrameric GINS complex stably associate with MCM2-7 with the assistance of accessory proteins to form the replicative CMG helicase that unwinds the parental DNA duplex during replication (Ilves et al., 2010). Although many origins are licensed, most do not fire during normal S-phase, but rather serve as backup origins for dormant origin firing (Blow et al., 2011; Ge et al., 2007; McIntosh and Blow, 2012; Woodward et al., 2006). At fired origins, however, two sister replication forks are formed by the activity of the CMG helicases, that bi-directionally unwind DNA at each origin. DNA synthesis is initiated by the Pol α complex which synthesizes the RNA primer, and is extended by the association of PCNA, RFC, and high fidelity replicative DNA polymerases ϵ and δ , which replicate DNA on the leading and lagging strands, respectively (Gaillard et al., 2015). During this entire process, the cell must balance accuracy, speed, and consumption of resources to ensure proper completion.

1.1.2. Replication Stress and Its Sources

Unfortunately, DNA replication forks are persistently challenged and arrested by endogenous and exogenous insults. These insults block the progression of the replisome and induce a phenomenon described as replication stress. Although the exact definition of replication stress remains ambiguous, as this phenomenon can arise from varying sources, and result in a

multitude of repercussions, it is characterized as “the slowing or stalling of the replication fork and/or DNA synthesis” (Berti and Vindigni, 2016; Gaillard et al., 2015; Zeman and Cimprich, 2014). Importantly, replication stress is the primary cause of genomic instability (Aguilera and García-Muse, 2013). The sources of replication stress are quite diverse (Zeman and Cimprich, 2014). The most commonly recognized source is unrepaired DNA lesions that act as physical barriers that block the progression of the replication machinery. These lesions include by-products of cellular metabolism, photo by-products, chemical mutagens, and reactive aldehydes. In addition to physical lesions, misincorporation of ribonucleotides during synthesis by replication polymerases also induces replication stress. Although replicative polymerases pol ϵ and pol δ typically have high fidelity base pairing, they are less able to distinguish dNTPs from rNTPs and actually misincorporate rNTPs at a surprisingly high rate (Dalgaard, 2012; Nick McElhinny et al., 2010). Types of DNA sequences such as repetitive sequences, common fragile sites (CFS), and G-quadruplexes that form non-B DNA structures also challenge the replisome (Barlow et al., 2013; Bochman et al., 2012; Paeschke et al., 2013). Moreover, concomitant replication and transcription are also sources of replication stress, not only through the physical collisions of the machineries involved but also by the torsional stress generated from these simultaneously occurring processes (Bermejo et al., 2012; Helmrich et al., 2013). Loss of RNA processing (Huertas and Aguilera, 2003; Li and Manley, 2005; Stirling et al., 2012), limited nucleotide pools or replication machinery components (Anglana et al., 2003; Bester et al., 2011; Poli et al., 2012; Zeman and Cimprich, 2014), levels of histones and histone chaperones (Aguilera and García-Muse, 2013), RNA-loops (Aguilera and García-Muse, 2012; Gaillard and Aguilera, 2016), and oncogene activation (Gaillard et al., 2015) all contribute to the generation of replication stress as well.

The resulting replication stalling may be transient or persist. Stalled forks result in the formation of stretches of single-stranded DNA (ssDNA) either through the physical uncoupling of

the helicase from DNA polymerases or through another process that involves nucleases and helicases or translocases actively processing stalled forks and creating ssDNA at fork junctions (Berti and Vindigni, 2016; Byun et al., 2005; Lopes et al., 2006; Pagès and Fuchs, 2003; Zellweger et al., 2015). ssDNA is rapidly coated by replication protein A (RPA) and forms primer-template junctions. These structures serve as signaling platforms that stimulate ATR/Chk1 activation, promoting recruitment of replication stress-response proteins that stabilize the fork (MacDougall et al., 2007; Nam and Cortez, 2011; Zou and Elledge, 2003). Defects in the replication stress response or prolonged fork stalling can result in replication fork collapse and generation of DNA breaks. These outcomes serve as a major mechanism for genomic instability, leading to tumorigenesis and other toxic consequences. To avoid these problems, cells developed DNA damage tolerance mechanisms that restart the stalled forks by bypassing these lesions.

1.1.3. Cellular Mechanisms for Relieving Replication Stress

To avoid genomic threat, the cell has evolved to develop a complex set of mechanisms to address and resolve obstructions that interfere with the faithful completion of DNA replication (Hanawalt, 2015). These mechanisms are described as fork restart and DNA damage tolerance (DDT) mechanisms (Fig 1) that enable a cell to tolerate and bypass sources of replication stalling such as DNA damage and other obstructions. One mechanism the cell utilizes is the firing of dormant origins (Fig 1A). Surprisingly, most licensed origins do not fire during S phase and remain dormant (McIntosh and Blow, 2012). However, under replication stress, dormant origins that are in proximity to the stalled site are activated to ensure complete replication in a timely manner. How dormant origins are fired and whether the initiation is passive or active remains to be elucidated.

The cell can also use post-replicative repair (PRR) (Fig 1B), that permits the replication machinery to skip damaged DNA and re-prime the fork downstream of the lesion to restart replication, leaving behind a ssDNA gap to be repaired after replication. These gaps are usually filled by the potentially error-prone translesion synthesis (TLS) pathway or a lesser-understood error-free template switching pathway. TLS is a major mechanism that the cell uses to replicate across DNA lesions (Lange et al., 2011). TLS is promoted by PCNA monoubiquitination, a modification that recruits specialized low-fidelity TLS polymerases (pol η , Rev1, pol κ , pol ι , pol ν , Rev3L-Rev7, and pol θ) that can incorporate nucleotides across the lesion. Not only can this mechanism occur post-replication (Fig 1B), it can also occur in real time (Fig 1C) where, at the stalled fork, a DNA polymerase is switched for a TLS polymerase to replicate across the obstacle, and switched back to a replicative polymerase after synthesis across the obstacle is complete. The alternative error-free template switching relies on a homology-directed repair mechanism, where the newly synthesized strand of the sister DNA duplex is used as a template (Mailand et al., 2013).

Fork reversal (Fig 1D) is another DNA damage tolerance (DDT) mechanism that promotes fork restart (Branzei and Szakal, 2016; Neelsen and Lopes, 2015). Here, the stalled replication fork reverses its course and rewinds the parental DNA to remodel the replication fork into a four-way “chicken foot” structure and assist in damage repair and fork restart. This pathway is not well understood, but is emerging as a critical process that essentially acts as a cellular “emergency brake” to pause DNA replication and provide time, room, and the correct DNA template before restarting the stalled fork (Neelsen and Lopes, 2015). This mechanism is achieved by a two-step process. First, the coordinated annealing of two newly synthesized strands creates a reversed fork. Second, the reversed fork is restarted. In some cases, fork backtracking and controlled limited degradation of nascent DNA can promote efficient fork restart (Costanzo, 2011).

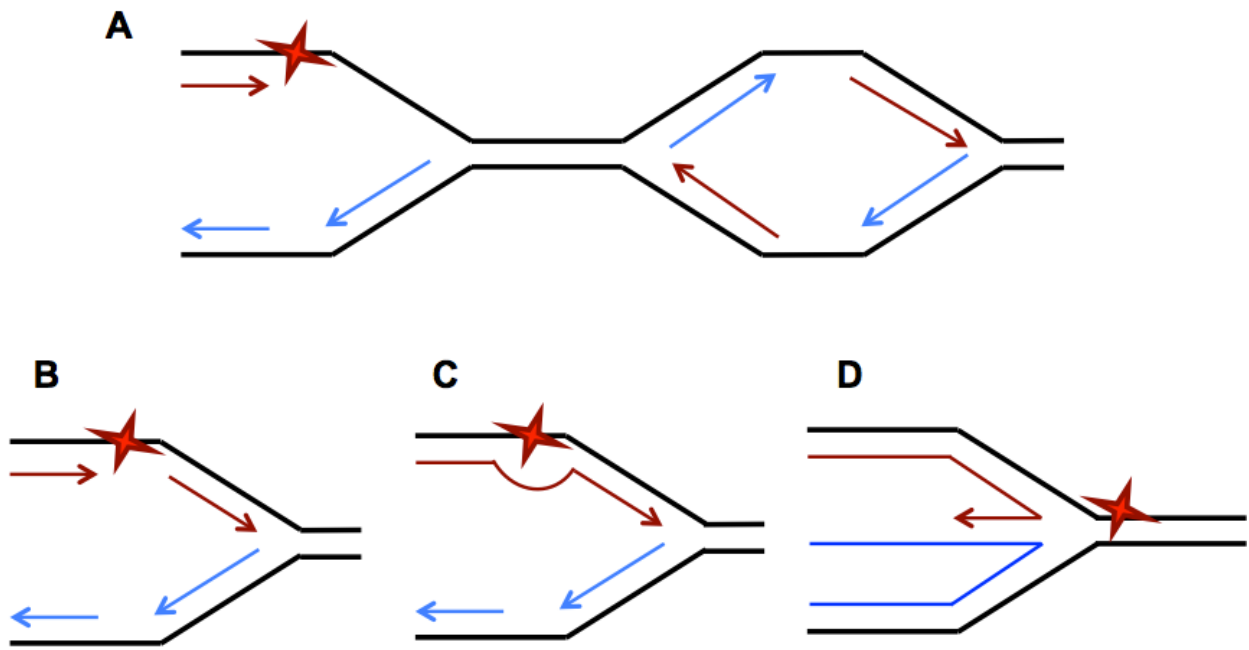


Figure 1. DNA damage tolerance and fork restart mechanisms. A. Dormant origin firing. At a stalled fork, dormant origins proximal to the stalled site will fire to complete replication. B. Post-replicative repair. The replication machinery skips the stalled site and reprimers replication downstream to continue with DNA synthesis. C. TLS in real time. At the stalled site, DNA polymerase will be switched for a TLS polymerase to replicate across the obstruction. Afterwards, the TLS polymerase will be switched back for a DNA polymerase to continue with replication. D. Fork reversal. At a stalled site, the stalled fork reverses its course and rewinds parental DNA and forms a “chicken foot structure.”

How the cell chooses between the different fork restart mechanisms is still unknown. If fork restart fails through any of these mechanisms, the fork is likely to collapse and result in passive or active nucleolytic generation of DNA breaks, leading to genomic instability and pathological consequences.

1.1.4. Consequences of Replication Stress

The consequences of replication stress manifest as heterogeneous phenotypes that give rise to mutations, cancer, aging, and other diseases. Cancer is the most common human disease associated with replication stress (Aguilera and García-Muse, 2013). Other standard pathological consequences that are well-known to be associated with replication stress include neurodegeneration, premature aging, mental retardation, growth retardation, and seizures. In

addition to these standard pathologies, atypical pathologies that result from replication stress have also been described. These uncharacteristic phenotypes include microcephalic primordial dwarfism, ciliopathies, and laminopathies (Zeman and Cimprich, 2014). These outcomes suggest that replication stress has a broader impact in human disease than initially assumed, and highlights the importance of studying how the cell addresses sources of replication stress to avoid toxic outcomes and preserve genomic integrity.

1.2. PCNA IN REPLICATION AND REPAIR

1.2.1. General Structural and Functional Characteristics of PCNA

PCNA (proliferating cell nuclear antigen) is a ring-shaped homotrimer that encircles the DNA (Fig 2), hence it is also referred to as a sliding clamp (Boehm et al., 2016a; Dieckman et al., 2012; Moldovan et al., 2007; Warbrick, 2000). PCNA has a highly conserved six-fold symmetry since each of the monomers are composed of two similarly folded globular regions united by a flexible interdomain connecting loop (IDCL). The face of PCNA pointing in the direction of DNA synthesis is known as the front face, and it is the site of interaction for most binding partners. A staggering number of proteins are shown to interact directly with PCNA. Thus, they must compete with each other for dancing with the sliding clamp (De Biasio and Blanco, 2013; Maga and Hubscher, 2003; Mailand et al., 2013). Most interacting partners bind PCNA through a conserved sequence termed the PIP (PCNA-interacting peptide)-box, which inserts itself into a hydrophobic pocket on the front face, beneath the IDCLs. The consensus PIP sequence is Q-x-x-Ψ-x-x-θ-θ, in which Ψ is a moderately hydrophobic amino acid (L, V, I, or M) and θ is an aromatic residue (Y or F). Many partners have degenerate sequences missing some of the core amino acids, and are still able to bind PCNA. A reverse PIP-box is also able to support PCNA interaction (Pedley et al., 2014). In addition, a second PCNA interacting motif, termed APIM

(AlkB homologue 2 PCNA-interacting motif) is widespread among DNA repair proteins and is defined as K/R-F/Y/W-L/I/V/A-L/I/V/A-K/R (Gilljam et al., 2009). The APIM interaction surface on PCNA partially overlaps with that used by the PIP-box.

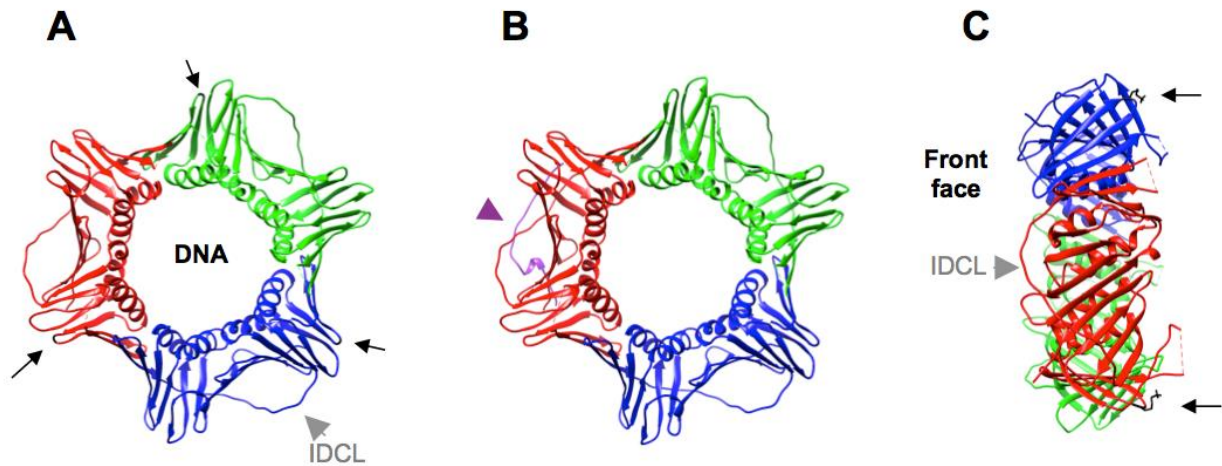


Figure 2. PCNA structure. A. Front view of PCNA. Each monomer is presented in a different color. Arrows indicate the position of K164, the residue targeted by ubiquitination and SUMOylation. Grey arrowhead indicates the interdomain connecting loop (IDCL) on one of the monomer. **B.** Front view of PCNA showing the interaction with a p21-derived PIP-box peptide (in purple, marked by arrowhead). **C.** Side view. Arrows indicate K164 residue on two of the monomers. Grey arrowhead indicates the IDCL on one of the monomers. The Front face is also indicated.

The diversity of sequence variations of PCNA binding domains (Table 1) allows for differential binding strengths. For example, the specialized PIP box Q-x-x-Ψ-T-D-θ-θ provides a higher affinity for PCNA (Havens and Walter, 2009). This indicates that the PIP-box motifs lacking TD are suboptimal, since they provide lower binding affinity. The TD-containing PIP-box of the CDK inhibitor p21 is one of the strongest PCNA-interacting peptide (Bruning and Shamoo, 2004). This ability to finely tune the interaction affinity through residues other than those conserved in the consensus sequence provides the first level of regulation of the competition for PCNA binding. Indeed, modifying the PIP-box of crucial replication and repair proteins to increase the strength of PCNA interaction results in impaired replication and repair (Fridman et al., 2010), indicating that competition through binding strength is a critical regulatory mechanism.

<i>Interaction motif</i>	<i>Sequence</i>	<i>Example</i>
PIP-box	Q x x L/V/I/M x x F/Y F/Y	QRSIMSFF (in DNA Ligase I)
Specialized PIP-box for strong interaction	Q x x L/V/I/M T D F/Y F/Y	QTSITDFF (in p21)
PIP-degron	Q x x L/V/I/M T D F/Y F/Y x x x K/R	QRRVTDFFARRR (in CDT1)
Inverted PIP-box	F/Y F/Y x x L/V/I/M M x x Q	FFAGIVWQ (in Akt)
APIM	K/R F/Y/W L/I/V/A L/I/V/A K/R	NKFLARE (in RAD51B)

Table 1. PCNA interacting motifs. The consensus residues are shown in red.

PCNA is an essential co-factor for DNA polymerases during replication (O'Donnell et al., 2013; Siddiqui et al., 2013). PCNA tethers polymerases to DNA and dramatically increases their processivity (the average number of nucleotides added before dissociation from DNA). Because of this activity, PCNA is essential for viability in all organisms. PCNA also participates in non-replicative DNA synthesis events, such as those occurring during DNA repair. Repair DNA synthesis events are common to a variety of DNA repair processes including nucleotide excision repair, homologous recombination, and mismatch repair. Moreover, the functions of PCNA extend well beyond DNA synthesis. While devoid of enzymatic activity itself, PCNA exerts a strong influence on the metabolism of DNA and chromatin by recruiting various enzymes to DNA. PCNA not only participates in localizing these factors to their sites of action but, in many instances, it also directly activates their enzymatic activities.

The repertoire of PCNA functions is dramatically amplified by its post-translational modifications, varying from small groups such as phosphorylation to whole proteins as in the case of ubiquitination (Hoegge et al., 2002; Moldovan et al., 2007). Since PCNA lacks enzymatic activity and instead exerts its effects through protein-protein interactions, regulated addition and removal

of these moieties to PCNA provides an ideal mechanism for controlling PCNA functions. These modifications generally do not affect PCNA structure. Instead, they provide an additional surface for interaction with specific binding factors –the second level of regulating PCNA-interactions. This is the case even for bulky modifications such as ubiquitination or addition of the ubiquitin-like protein SUMO (Tsutakawa et al., 2015). Structures of mono-ubiquitinated PCNA using X-ray crystallography, electron microscopy, and Small angle X-ray scattering (Lau et al., 2015; Tsutakawa et al., 2015; Zhang et al., 2012) revealed several different conformations including one with ubiquitin extending away from PCNA, as well as a “docked” conformation in which ubiquitin interacts extensively with the PCNA surface forming a contiguous binding interface. It is possible that these different conformations allow ubiquitinated PCNA the necessary plasticity to interact with different binding partners. In contrast, SUMO is simply tethered to PCNA without additional interactions, resulting in an extended, flexible tag, which acts as an independent interaction module (Armstrong et al., 2012; Tsutakawa et al., 2015). As detailed below, PCNA post-translational modifications are generally associated with particular functional outcomes (Table 2).

<i>Modification</i>	<i>Target site</i>	<i>Enzyme</i>	<i>Outcome at the molecular level</i>	<i>Biological function</i>
Phosphorylation	Y211	EGFR, c-Abl (kinases)	Protects against PCNA degradation	Promotes cell proliferation
			Inhibits MutS binding	Inhibits MMR
Mono-ubiquitination	K164 (and potentially others)	RAD18, CDT2 (E3 ligases)	Recruits TLS polymerases	Promotes TLS
K63-linked multi-ubiquitination	K164	HLTF, SHPRH (E3 ligases)	Recruits the translocase ZRANB3	Promotes error-free bypass?
ISGylation	K168	EFP (E3 ligase)	Recruits USP10 to de-ubiquitinate PCNA	Turns off TLS

SUMOylation	K164	UBC9 (E2 conjugating enzyme; No E3 ligase necessary?)	Recruits HR inhibitor PARI	Inhibits HR
Acetylation	K13, K14, K20, K77, K80, K284	CBP, p300 (acetyl-transferases)	Promotes removal of PCNA from DNA after NER	Promotes genomic stability

Table 2. Post-translational modifications of human PCNA.

1.2.2. PCNA in DNA Replication

DNA replication is initiated at distinct replication origins, which are marked by binding of the pre-replicative protein complex in G1 phase of the cell cycle (O'Donnell et al., 2013; Siddiqui et al., 2013). Origin firing during S-phase involves the assembly of two back-to-back replication forks that proceed in opposing directions. Each replication fork is headed by the CMG helicase, which unwinds the DNA. This allows synthesis of the primer by the DNA Polymerase α complex, which contains one catalytic subunit responsible for synthesis of the initial ~10nt RNA primer, and a separate DNA polymerase subunit that extends the RNA primer with about ~20nt. PCNA is loaded at the primer-template junction by the multi-subunit Replication Factor C (RFC) complex, which can open the PCNA ring and clamp it on the DNA. While the fork assembly takes place on a DNA region devoid of nucleosomes, PCNA loading seems to require a chromatin context marked by methylation of histone H3 at lysine K56, which is catalyzed by the methyltransferase G9a (Yu et al., 2012). In vitro reconstitution of the replisome complex assembly showed that, in the presence of PCNA-RFC, the primase is switched with the high fidelity replicative DNA polymerases ϵ (on the leading strand) and δ (on the lagging strand) (Georgescu et al., 2014; Georgescu et al., 2015). On the leading strand, DNA synthesis catalyzed by pol ϵ proceeds continuously. PCNA interaction with pol ϵ is weak and PCNA may even dissociate and remain behind the fork, to mark the leading strand for post-replicative events such as mismatch repair.

Since DNA synthesis can only occur in the 5'-3' direction, replication on the lagging strand is discontinuous. On this strand, PCNA interacts with multiple PIP-box containing subunits of pol δ , as well as with the pol δ -associated protein PDIP46 (Wang et al., 2016). Primers are being synthesized every 100-200nt to generate Okazaki fragments. The end of the previous Okazaki fragment functions as a molecular break that slows down pol δ ten-fold. The polymerase partly displaces the primer end forming a flap structure that is cleaved off by FEN1, one nucleotide at a time (Stodola and Burgers, 2016). Eventually, DNA ligase I seals the resulting nick. Both enzymes interact with PCNA, which activates their activities (Zheng and Shen, 2011). Formation of a stable, active Okazaki fragment maturation complex in which different monomers of the PCNA trimer interact with pol δ , FEN1, and DNA ligase I respectively (a so-called "PCNA tool belt" - Fig 3) have been proposed many years ago, and recently demonstrated experimentally (Stodola and Burgers, 2016). On the other hand, mutant PCNA trimers with a single binding site are perfectly capable of supporting Okazaki fragment maturation (Dovrat et al., 2014) arguing that tool belts may not be absolutely required. Moreover, post-translational modifications of FEN1 seem to play an important role in regulating these interactions (Zheng and Shen, 2011).

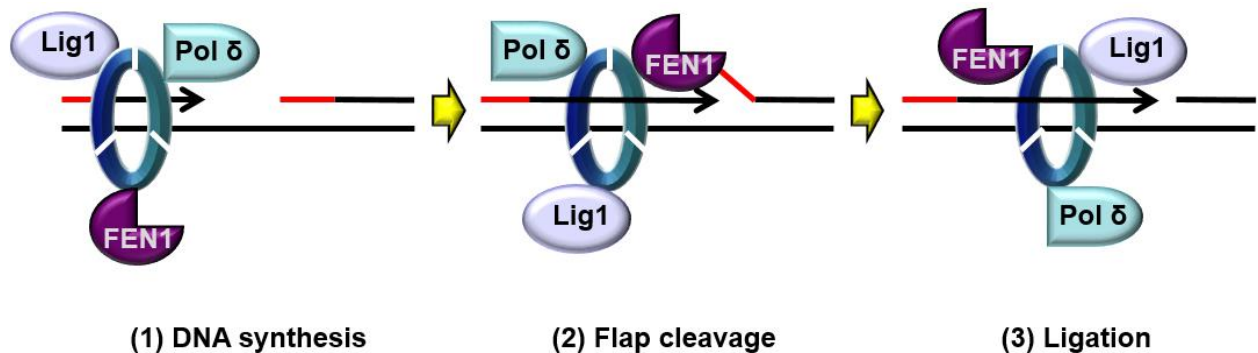


Figure 3. Example of PCNA tool-belt: the Okazaki fragment maturation complex. Pol δ , FEN1, and DNA Ligase I bind PCNA simultaneously and each interact with a monomer of the PCNA trimer to facilitate enzyme switching during Okazaki fragment maturation.

Because it is loaded on the primer template junction at each Okazaki fragment, PCNA accumulates on the lagging strand. Unloading of PCNA requires the activity of an RFC-like

complex in which the catalytic subunit RFC1 is replaced by ATAD5 (Elg1 in yeast) (Kubota et al., 2013; Lee et al., 2013). Loss of this unloading activity results in genomic instability, underlining the importance of removing PCNA from DNA (Johnson et al., 2016). Interestingly, Elg1 is itself inhibited during G1 to allow PCNA loading on DNA at the G1/S transition (Huang et al., 2016).

1.2.2.1. PCNA is a Co-Factor for Regulated Protein Degradation

During Replication

One essential activity of PCNA is to promote degradation of a subset of its binding partners. A large number of substrates have been identified for this PCNA-targeted degradation. The CDK inhibitors p21 and Xic1 are degraded to allow normal cell cycle progression (Abbas et al., 2008; Kim et al., 2010). Degradation of the accessory p12 subunit of pol δ alters its enzymatic activity (Zhang et al., 2013). The thymine DNA glycosylase TDG is degraded to prevent unwanted DNA demethylation (Shibata et al., 2014; Slenn et al., 2014). Degradation of the anti-recombinogenic FBH1 helicase regulates DNA repair (Bacquin et al., 2013). The replication licensing factors CDC6 and CDT1 are degraded to prevent re-initiation of DNA replication (re-replication) (Arias and Walter, 2006; Clijsters and Wolthuis, 2014). Degradation of the histone methyltransferase Set8 prevents untimely chromatin compaction (Centore et al., 2010; Oda et al., 2010). Degradation of these substrates occurs only when they are bound to PCNA on chromatin during S-phase or after DNA damage exposure, and requires ubiquitination of the substrate by the CRL4-CDT2 ubiquitin ligase. The PCNA interactors degraded through this mechanism possess a special type of PIP-box, termed PIP-degron: Q-x-x- Ψ -T-D- ϑ - ϑ -x-x-x-B, where B is a basic residue (K or R) (Havens and Walter, 2009). Importantly, this basic residue was shown to recruit the CDT2 ubiquitin ligase, but only when the substrate is bound to chromatin-loaded DNA. The mechanistic

basis for this is still unclear. Once ubiquitinated by CDT2, the substrates are then removed from chromatin by the ubiquitin-targeted chaperone p97, a hexameric AAA-ATPase complex known to remodel ubiquitin complexes, and delivered to the proteasome for degradation (Raman et al., 2011). In order to allow re-expression of the substrates in G2 phase, CDK1 phosphorylates CDT2 in late S-phase preventing its recruitment to chromatin-bound PCNA (Rizzardi et al., 2015).

1.2.2.2. PCNA and Chromatin Assembly

Nascent DNA is instantly coated with nucleosomes in a co-replicative process that requires binding of the histone chaperone CAF-1 to PCNA at replication forks (Zhang et al., 2000). Interestingly, chromatin assembly can regulate the speed of replication fork progression: reducing histone pools results in decreased replication speed. Under these conditions, PCNA is retained on chromatin to allow CAF-1 recruitment once histones become available (Mejlvang et al., 2014). Not only do nucleosomes need to be assembled on nascent DNA, but their histone marks also need to be preserved so that the maternal epigenetic status is maintained in the daughter cells. Parental histones ahead of the replication forks are relocated to nascent DNA behind the replication fork, providing a possible mechanism for transferring epigenetic modifications. However, it was recently shown that most histones loaded on the nascent DNA lack modifications present on the nucleosomes ahead of the fork, such as H3K4me3 or H3K27me3 modifications. Instead, epigenetic inheritance relies on the presence of the enzymes responsible for such modifications (histone methylation, acetylation, ubiquitination) in close proximity to PCNA during replication (Petruk et al., 2013; Petruk et al., 2012). It is likely that these enzymes interact with PCNA. Moreover, DNMT1, an enzyme responsible for DNA methylation at CpG islands, binds directly to PCNA (Mortusewicz et al., 2005). Finally, PCNA participates in setting up sister

chromatid cohesion, by recruiting cohesion establishing factors to replication forks (Moldovan et al., 2007).

1.2.2.3. Regulation of Cell Proliferation through PCNA

Because of its essential role in DNA replication, regulation of PCNA steady state levels represents an important mechanism to control proliferation in response to extracellular conditions. Growth factor signaling (EGF through EGFR kinase, and HGFL through c-Abl kinase) results in phosphorylation of PCNA at Tyr211, which increases its chromatin association and protects it from degradation (Wang et al., 2006; Zhao et al., 2014). Several other signaling molecules have been shown to regulate proliferation through controlling PCNA levels, including ERK8 (Groehler and Lannigan, 2010) and 14-3-3 ζ (Gao et al., 2015).

In addition to these signaling factors a significant subset of PCNA interactors are regulators of cell fate. One of the most potent proliferation inhibitors, namely p21, blocks not only CDKs but also PCNA, through a tight PIP-box mediated association that out-competes DNA polymerases and other interactors (Moldovan et al., 2007). In turn, PCNA can modulate apoptosis through interactions with p53 and other apoptotic regulators. For example, in mature non-proliferating neutrophils, PCNA accumulates in the cytosol where it binds to procaspases blocking their activation and inhibiting apoptosis (Witko-Sarsat et al., 2010).

1.2.3. PCNA in DNA Damage Tolerance

1.2.3.1. PCNA in Translesion Synthesis

One of the major activities of PCNA is to promote tolerance of DNA damage during DNA replication. DNA lesions block the progression of high fidelity replicative DNA polymerases δ and ϵ . To bypass them, cells employ specialized low fidelity polymerases (such as pol η , pol κ ,

and Rev1)—a process termed translesion synthesis (TLS) (Cipolla et al., 2016; Jansen et al., 2015). TLS truly is a double-edged sword: while bypass of DNA lesions allows cells to continue their proliferation program without replication arrest, low-fidelity polymerases are mutagenic on both normal and damaged DNA templates, and thus must be kept in check. The regulation of TLS relies on post-translational modification of PCNA (Bienko et al., 2005; Guo et al., 2006; Hoege et al., 2002; Kannouche et al., 2004; Plosky et al., 2006; Watanabe et al., 2004). Upon fork stalling at DNA lesions, PCNA mono-ubiquitination at Lys 164 recruits TLS polymerases to bypass the lesion (Fig 4). The molecular basis for this recruitment is the presence of separate PCNA-interacting domains and ubiquitin-interacting domains in most TLS polymerases, providing higher binding affinity for the ubiquitinated form of PCNA. In contrast, replicative DNA polymerases only possess PCNA-binding domains and lack ubiquitin-interacting domains. Indeed, it was recently shown that PCNA ubiquitination does not affect the assembly or processivity of the pol δ complex (Hedglin et al., 2016).

1.2.3.2.Fine-Tuning TLS Regulation

The various TLS polymerases differ in their abilities to bypass different lesions; some of them can even ensure accurate bypass of specific lesions, such as in the case of pol η -mediated bypass of UV-induced thymidine dimers. Mechanisms for specific recruitment of a polymerase to a lesion are not yet known. Recent models suggest that this may be a stochastic process: since PCNA is a trimer, tool belts can be formed in which each monomer binds a different polymerase, albeit with low affinity. This ensures a high local concentration of various TLS polymerases that can individually sample the lesion and eventually engage it (Boehm et al., 2016b). Such tool belts have been observed even in the absence of ubiquitination. Indeed, under certain conditions polymerases can perform TLS in the absence of PCNA ubiquitination (Acharya et al., 2010;

Despras et al., 2012; Hendel et al., 2011; Krijger et al., 2011b; Wit et al., 2015). However, PCNA ubiquitination not only increases efficiency, but also alters the mutation spectra of TLS (Hendel et al., 2011). It is likely that the addition of ubiquitin provides an additional molecular glue that the polymerases can attach to with varying affinities.

Turning off TLS is an important aspect of the pathway, since low fidelity TLS polymerases can wreak havoc on undamaged substrates. The deubiquitinating enzyme USP1 removes ubiquitin from PCNA thus promoting high fidelity DNA synthesis (Huang et al., 2006). Besides USP1-mediated deubiquitination, several other mechanisms have been described. The protein SPARTAN binds ubiquitinated PCNA at stalled forks and is important for maintaining ubiquitin-PCNA levels through a yet unclear mechanism (Centore et al., 2012; Ghosal et al., 2012; Juhasz et al., 2012). At the same time, SPARTAN plays a crucial role in switching off TLS, by recruiting the ubiquitin-selective chaperone p97 to remove pol η from DNA (Davis et al., 2012; Mosbech et al., 2012). The small PCNA interacting protein PAF15 also participates in turning off TLS by tightly associating with PCNA following lesion bypass, out-competing TLS polymerases (De Biasio et al., 2015; Povlsen et al., 2012).

TLS is also regulated by an additional PCNA modification. Recently, it was shown that after DNA damage, PCNA is modified with the ubiquitin-like molecule ISG15 (ISGylation) through the recruitment of the ISG15 E3 ligase EFP to ubiquitinated PCNA. This modification then recruits the deubiquitinating enzyme USP10 to remove ubiquitin from PCNA and restore high fidelity DNA synthesis (Park et al., 2014).

Additional layers of complexity have been added in recent years. The ubiquitin ligase CDT2 can also ubiquitinate PCNA at K164 to promote TLS, but unlike RAD18, it seems to be associated with endogenous replication stress rather than DNA damage exposure (Terai et al., 2010). PCNA ubiquitination can also be increased by a variety of factors, including: the repair

proteins FANCD2 (Chen et al., 2016) and PTIP (Gohler et al., 2008), the ADP-ribosyltransferase PARP10 (Nicolae et al., 2014), the signaling molecules SIVA1 (Han et al., 2014), and MAGE-A4 (Gao et al., 2016), the ubiquitin ligases BRCA1 (Tian et al., 2013) and RNF8 (Zhang et al., 2008), the checkpoint proteins CHK1 and claspin (Yang et al., 2008), and others. While it is unclear how each of these factors fit in the bigger picture, they likely provide fine-tuning mechanisms ensuring that the proper balance of TLS is achieved, depending on cell type, growth conditions and proliferation signals, cell cycle stage, chromatin status, amount of damage etc.

1.2.3.3. Timing of PCNA Ubiquitination and TLS

PCNA ubiquitination is normally detected during S-phase in both yeast and human cells (Hoegge et al., 2002; Huang et al., 2006; Terai et al., 2010). Mechanistic studies have shown that uncoupling between the helicase machinery and the stalled polymerase results in accumulation of single stranded DNA that recruits RAD18 to ubiquitinate PCNA (Chang and Cimprich, 2009; Davies et al., 2008). Moreover, chromatin remodeling by the ZBTB1-KAP1 complex promotes RAD18 recruitment and PCNA ubiquitination, perhaps by increasing RAD18 accessibility to DNA (Kim et al., 2014). Switching the replicative polymerase with a TLS polymerase seems to be a straightforward and time-saving mechanism for restarting a stalled replication fork and continuation of DNA replication. Thus, PCNA ubiquitination and TLS were thought to occur co-replicationally, taking place instantaneously following fork stalling –a process sometimes referred to as “on the fly” (Hedglin and Benkovic, 2015; Yang et al., 2013a). While there is evidence for PCNA ubiquitination-mediated polymerase switching *in vitro* (Masuda et al., 2010), the process can occur independent of PCNA ubiquitination *in vivo*. (Edmunds et al., 2008). Consistent with this, genetic experiments in yeast showed that PCNA ubiquitination can also occur in G2 phase, without impeding on damage tolerance (Daigaku et al., 2010; Karras and Jentsch, 2010). These

results would instead point towards a post-replicative model for TLS, where the fork is re-established downstream of the lesion leaving behind a short unreplicated region with the lesion, to be repaired or bypassed at a later time in S-phase or even G2 (Figure 4).

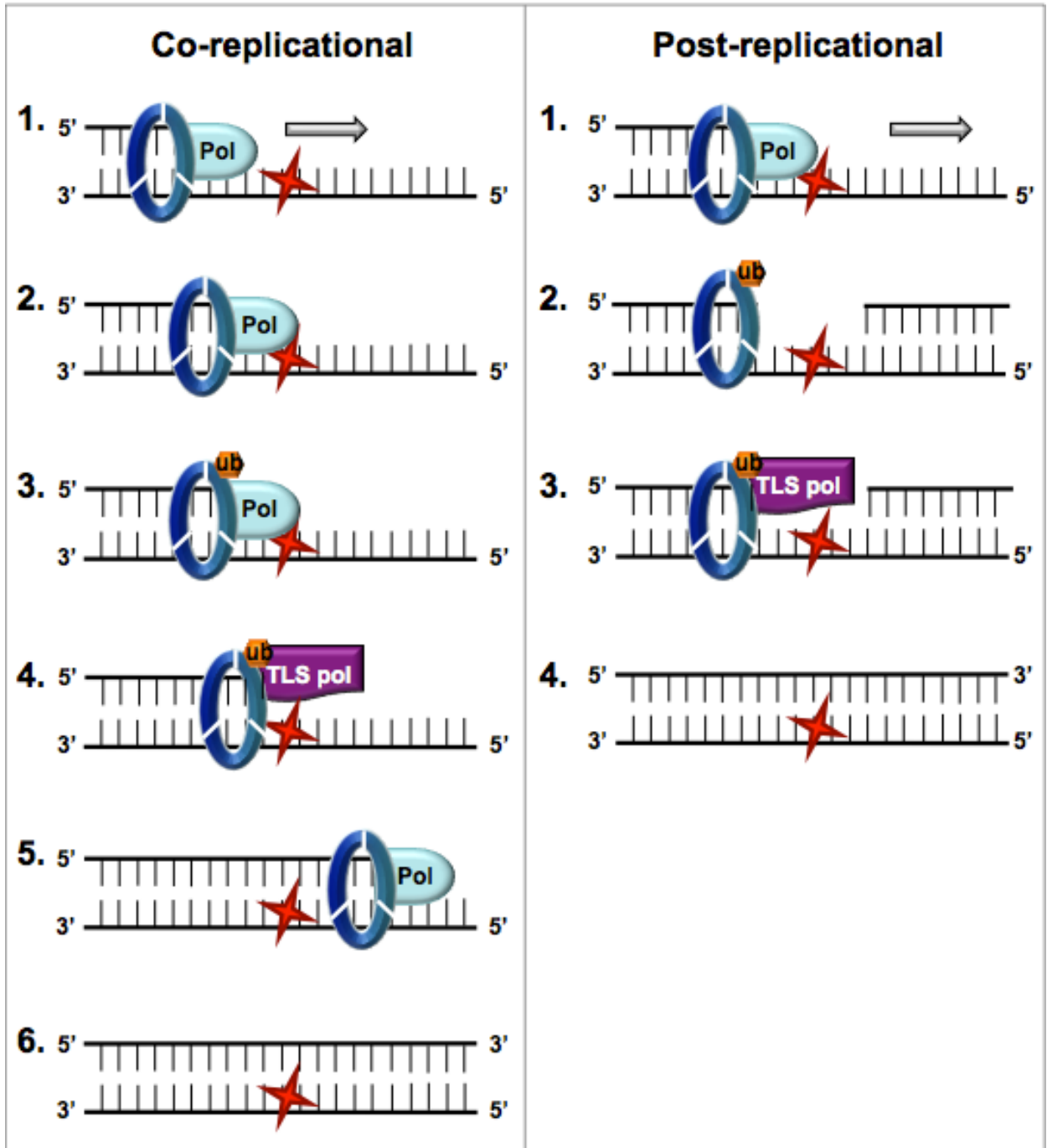


Figure 4. Two non-exclusive models for the timing of TLS. In the co-replication model, also termed “on-the-fly,” the polymerase switch occurs instantaneously upon stalling, following PCNA ubiquitination. In the post-replicative model, the fork is reassembled downstream of the lesion leaving behind a gap which is filled later in S phase or G2 by TLS polymerases. Whether PCNA is left behind at the gap or is loaded *de novo* is not clear.

Mechanistic details of such a process are missing and many important questions remain unanswered: Is the integrity of the stalled fork maintained? Is PCNA ubiquitinated upon stalling or rather later, right before bypass? Why resort to bypass when behind the fork, the lesion could be repaired error-free for example through recombination? Nevertheless, such a post-replicative mechanism would provide the cell with additional flexibility to deal with a larger number of lesions, and would perhaps provide the opportunity to sample the local environment of each individual lesion before deciding on bypass versus repair. The two possibilities may not be mutually exclusive (Waters et al., 2009). Interestingly, p53 was recently shown to bind PCNA at stalled forks and suppress the extension from these forks (Hampp et al., 2016), perhaps providing a mechanism for marking those particular stalled forks which will be resolved post-replicationally.

1.2.3.4. Non-Canonical Roles of PCNA Ubiquitination

PCNA ubiquitination can be detected outside of S-phase also in human cells, under certain conditions. For example PCNA ubiquitination can be induced by UV in quiescent (G0-arrested) cells (Ogi et al., 2010; Yang et al., 2013b). This event seems to promote recruitment of the TLS Pol κ to UV lesions (Ogi et al., 2010). Moreover, oxidative damage also induces PCNA ubiquitination outside S-phase (Zlatanou et al., 2011). While still dependent on RAD18, this ubiquitination event involves a non-canonical activity of the mismatch repair complex MSH2-MSH6, which is required to initiate removal of the damaged strand by the nuclease EXO1. In case DNA lesions are present on the remaining strand, which may frequently be the case, PCNA ubiquitination promotes a TLS-like lesion bypass event using low fidelity polymerases. Interestingly, even though this process still uses the canonical RAD18 ubiquitin ligase, an

alternative deubiquitinating enzyme, namely USP7, is employed to remove ubiquitin from PCNA under these conditions (Kashiwaba et al., 2015).

PCNA ubiquitination was recently shown to participate in activation of a novel replication stress checkpoint, which is mediated by ATM (unlike the classic replication stress checkpoint activated by ATR). Ubiquitinated PCNA recruits WRNIP protein, which in turn binds to the ATM activating cofactor ATMIN. This pathway is important for coordinating replication completion with cell cycle progression and, in its absence cells enter mitosis with under-replicated DNA (Kanu et al., 2016). Finally, recent genetic experiments in yeast indicated a role for PCNA ubiquitination and TLS in Okazaki fragment maturation, at least in the context of FEN1 inactivation (Becker et al., 2015).

1.2.3.5. PCNA Polyubiquitination

The PCNA ubiquitination events described above to regulate TLS are mono-ubiquitination events. However, PCNA can also be ubiquitinated by K63-linked multi-ubiquitin chains at Lys 164. This modification is significant in yeast, but present at much lower levels in human cells (Hoege et al., 2002). PCNA polyubiquitination is believed to initiate an alternative, error-free lesion bypass mechanism involving the use of the newly replicated sister chromatid as template; although proposed a long time ago, mechanistic details are still missing. The ZRANB3 translocase is recruited to poly-ubiquitinated PCNA and remodels the DNA structure at the stalled fork (Ciccio et al., 2012; Weston et al., 2012; Yuan et al., 2012), but how this promotes error-free lesion bypass is unclear. Further complicating the picture, the E3 ubiquitin ligases SHPRH and HLTF that are involved in PCNA poly-ubiquitination (Motegi et al., 2008), also participate in TLS (Lin et al., 2011).

1.3. HUWE1 IN THE CELL

1.3.1. *The E3 Ubiquitin Ligase*

Ubiquitin signaling is involved in a diverse set of cellular processes including the DNA damage response, development, neuronal expansion, cell division, cell fate, and immune signaling (Bernassola et al., 2008; Cipolla et al., 2016; Scheffner and Kumar, 2014; Scheffner and Staub, 2007; Vittal et al., 2015). Ubiquitin signaling involves the covalent attachment of ubiquitin to a substrate through a series of three sequential steps performed by: 1) E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase) (Scheffner et al., 1995). In humans, there are two known E1s, approximately thirty-five E2s, and hundreds of E3s that are responsible for attaching a single ubiquitin moiety or differently-linked chains of ubiquitin to thousands of protein substrates.

E3 ligases are primarily responsible for mediating substrate specificity of ubiquitin conjugation and either assist or directly catalyze the transfer of ubiquitin onto a substrate. E3 ligases constitute a large class of proteins, with over six hundred putative E3 ligases or E3 ligase complexes encoded by the human genome (Scheffner and Kumar, 2014). This group of enzymes are categorized into three classes: 1) homologous to E6-AP carboxyl terminus (HECT) E3 ligases, 2) really interesting new genes (RING) E3 ligases, and 3) ring between ring (RBR) E3 ligases. While all three classes carry out the final step of covalent ubiquitination of target substrates, they differ in their structure and mechanism. The HECT domain of HECT E3s possesses enzymatic function and is able to directly catalyze the covalent attachment of ubiquitin to substrate proteins through a ubiquitin-HECT thioester complex intermediate. Conversely, the RING domain of RING E3s lacks enzymatic activity and, instead, acts as an allosteric activator of the E2 ubiquitin-conjugating enzyme. In between HECT and RING E3s are the RBR ligases, which act as a hybrid

between HECT and RING enzymes. One RING of RBR ligases serves as a docking site for an E2 ubiquitin-conjugating enzyme, while the other RING accepts ubiquitin from the E2 through the formation of a thioester complex.

1.3.2. HUWE1: An Evolutionarily Conserved HECT E3 Ubiquitin Ligase

HECT E3 ubiquitin ligases were the first of the E3 classes to be identified (Huibregtse et al., 1995). This class is found in all eukaryotic organisms, and approximately 28 HECT E3 ligases are encoded by the human genome (Scheffner and Staub, 2007). HECT proteins are characterized by a C-terminal HECT domain that spans approximately 350 amino acids and harbors a cysteine residue that forms a catalytic thiol ester with ubiquitin (Bernassola et al., 2008). The HECT domain derived its name by sharing striking similarity with the C-terminus of E6-AP, a cellular protein that binds to E6 protein of oncogenic human papilloma virus and ubiquitinates tumor suppressor protein p53. While the HECT domain represents the catalytic domain, the substrate specificity of HECT E3 ubiquitin ligases are determined by their respective N-terminal extensions.

HUWE1 (also known as ARF-BP1, HECTH9, MULE, and Lasu1) is an evolutionarily conserved E3 ligase of the HECT family (Bernassola et al., 2008; Chen et al., 2005). This giant protein (482 kDa) is comprised of 4374 amino acids organized into several conserved domains. At the N terminus, HUWE1 contains two tandem armadillo repeat-like domains (ARLD) 1 and 2 (residues 104-374 for domain 1, residues 424-815 for domain 2), of unknown functions. Following the ARLDs is a ubiquitin-associated domain (UBA; residues 1317-1355), a small sequence motif commonly found in proteins that are linked to the ubiquitination pathway. In addition, HUWE1 contains a protein-protein interaction domain (WWE; residues 1612-1692), a Bcl-2 homology 3 domain (BH3; residues 1972-1994), a nuclear localization signal (NLS; residues 2236-2255)

domain and, at its C-terminus, the conserved catalytic HECT (residues 4016-4374) domain (Fig 5).

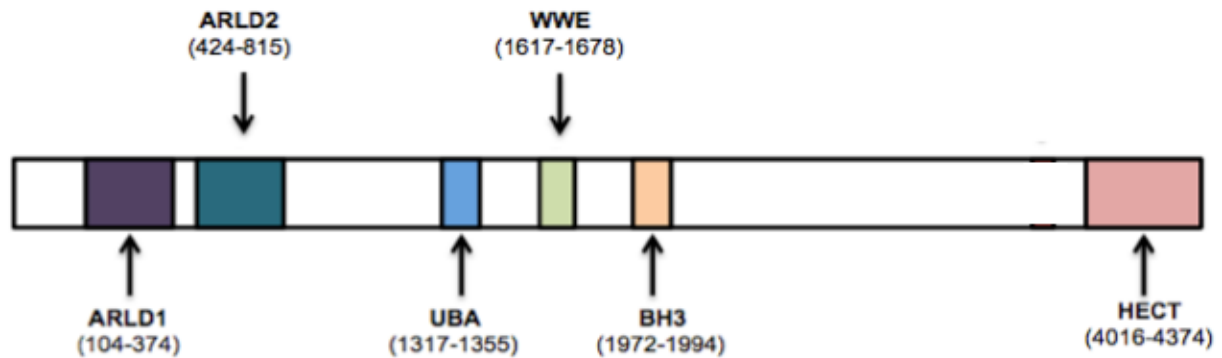


Figure 5. Full-length HUWE1 and its conserved domains. ARLD: armadillo repeat-like domain; UBA: ubiquitin-associated domain; BH3: Bcl2-homology 3 domain; HECT: homologous to the E6-AP carboxyl terminus (catalytic ubiquitin ligase domain).

HUWE1 was discovered by three independent groups, each of which reported a novel individual substrate for HUWE1 ubiquitination: Lys48-linked ubiquitination and targeted degradation of p53 to promote cellular homeostasis (Chen et al., 2005); Lys63-linked ubiquitination and functional modification of c-Myc to promote cellular proliferation (Adhikary et al., 2005); and Lys48-linked ubiquitination and targeted degradation of Mcl-1 to promote cell death (Zhong et al., 2005). HUWE1 regulates diverse cellular functions because of its promiscuity as an E3 ligase. HUWE1 has been shown to play important roles in regulating cell proliferation, apoptosis, development, tumorigenesis, neuronal differentiation, and DNA repair by acting on different substrates. The diversity of HUWE1 substrates includes but is not limited to: histones (Liu et al., 2005) and histone deacetylase HDAC2 (Yin et al., 2010; Zhang et al., 2011), circadian heme receptor Rev-erba (Yin et al., 2010), mitochondrial protein Mitofusin 2 (Leboucher et al., 2012), Atoh1 (Cheng et al., 2016), Shoc2 (Jang et al., 2014), muscle protein TBP (Li et al., 2015), Rac activator TIAM1 (Vaughan et al., 2015) and Wnt-signaling protein dishevelled (de Groot et al., 2014) (Table 3).

Target substrate	Type of modification	Effect	Source
p-53	K-48 ub	Promote degradation	Chen et al, Cell 2005
Myc	K-63 ub	Promote gene activation	Adhikary et al, Cell 2005
Mcl-1	Mono-ub	Promote K48-linked ub and degradation	Zhong et al, Cell 2005
Core histones	K-48 ub	Promote degradation	Liu et al, MCB 2005
Cdc6	K-48 ub	Promote degradation	Hall et al, MBC 2007
TopBP1	K-48 ub	Promote degradation	Herold et al, EMBO 2008
PoI β	K-48 ub	Promote degradation	Parson et al, EMBO 2009
Miz1	K-48 ub	Promote degradation	Yang et al, PNAS 2010
PoI λ	K-48 ub	Promote degradation	Markkanen et al, PNAS 2012
PP5	K-48 ub	Promote degradation	Kurokawa et al, Science Signaling, 2013
BRCA1	K-48 ub	Promote degradation	Wang et al, BBRC 2013
Dishevelled	K-63 ub	Inhibit Dvl multimerization, negatively regulate wnt signaling	Reinoud et al, Science Signaling 2014
Atoh1	K-48 ub	Promote degradation	Forget et al, Developmental Cell 2014
DDIT4	K-48 ub	Promote degradation	Thompson et al, JBC 2014
Shoc2	K-48 ub	Promote degradation	Jang et al, MCB 2014
TIAM1	K-48 ub	Promote degradation	Vaughan et al, Cell Reports 2015
TBP	K-48 ub	Promote degradation	Li et al, ELife 2015
H2AX	Mono-ub	Promote phosphoryation	Choe et al, EMBO Rep 2016
HDAC2	K-48 ub	Promote degradation	Zhang et al, Genes Dev 2011
Rev-erba	K-48 ub	Promote degradation	Yin et al, PNAS 2010

Mitofusin 2	K-48 ub	Promote degradation	Leboucher et al, Mol Cell 2012
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Table 3. HUWE1 substrates and outcomes of ubiquitination.

HUWE1 also regulates DNA repair through its target substrates. It has been reported that HUWE1 targets the checkpoint proteins CDC6 (Hall et al., 2007), TopBP1, and Miz1 (Herold et al., 2008; Inoue et al., 2013); the base excision repair polymerases β and λ (Khoronenkova and Dianov, 2011; Markkanen et al., 2012; Parsons et al., 2009); and the homologous recombination factor BRCA1 (Wang et al., 2014) for degradation. CDC6 is a highly conserved protein that is an essential component of the pre-RC, and is also involved in the ATR/Chk1 checkpoint signaling. HUWE1 regulates the cellular pool of CDC6 that is not bound to chromatin by adding Lys48-linked ubiquitin moieties and targeting CDC6 for degradation by the proteasome, thus regulating origin firing and checkpoint activation (Hall et al., 2007). HUWE1 also regulates checkpoint activation by acting on ATR kinase-activating protein TOPBP1 under UV irradiation, as well as its partner protein Miz1 (Herold et al., 2008). Pol β is involved in the base excision repair (BER) pathway, and HUWE1 regulates the cytoplasmic pool of pol β by targeting free pol β for degradation, thus negatively regulating BER (Parsons et al., 2009). Furthermore, HUWE1 negatively regulates the homologous recombination pathway by promoting BRCA1 degradation (Wang et al., 2014). Adding to this repertoire, most recently HUWE1 was described to regulate steady state levels of DNA damage signaling protein H2AX under double strand break-inducing agents such as γ irradiation (Atsumi et al., 2015). These reports describe HUWE1 as a negative regulator for DNA repair and checkpoint signaling.

The exact role of HUWE1 in genome stability is ambiguous. HUWE1 has been characterized as both a tumor suppressor and an oncogenic protein (de Groot et al., 2014; Hao et al., 2012; Inoue et al., 2013; Jang et al., 2014; Kurokawa et al., 2013; Maltseva et al., 2013; Pervin

et al., 2011; Peter et al., 2014; Qi et al., 2013; Schaub and Cleveland, 2014; Vaughan et al., 2015). HUWE1 is overexpressed in various cancer cell lines and HUWE1 mutations have been found in many cancers including lung, stomach, breast, colorectal, hepatic, and brain carcinomas (Adhikary et al., 2005; Chen et al., 2005; Confalonieri et al., 2009; Liu et al., 2012; Yoon et al., 2005; Zhao et al., 2008). These findings suggest that HUWE1 promotes tumorigenesis. This is further supported by HUWE1 promoting oncoprotein activation and cellular proliferation through c-Myc (Adhikary et al., 2005) and N-Myc (Zhao et al., 2009), as well as its role in promoting the degradation of tumor suppressor p53 (Chen et al., 2005) and DNA repair proteins. Paradoxically, HUWE1 also demonstrates tumor-suppressive properties, reflected by its substrate specificity for the anti-apoptotic protein Mcl-1 (Zhong et al., 2005) and its role in suppressing Ras-driven tumorigenesis in a mouse model by preventing c-Myc/Miz1-mediated downregulation of p21 and p15 (Inoue et al., 2013).

This thesis focuses on the characterization of a surprising and novel role for HUWE1 in preserving genomic stability by promoting tolerance to replication stress. We found that HUWE1 contains a PIP-box motif, and directly interacts with PCNA, which is essential for replication fork stability and genomic integrity. Moreover, we show that HUWE1 monoubiquitinates H2AX, a key protein involved in DNA damage signaling and the recruitment of repair proteins to damaged DNA. Overall, our findings demonstrate that HUWE1 is a novel regulator of replication stress, and an important player in genome maintenance.

Chapter 2. MATERIALS AND METHODS

2.1. CELL CULTURE AND PROTEIN TECHNIQUES

Human HeLa (ATCC# CCL2), 293T (ATCC# 3216), U2OS (ATCC# HTB-96), MCF7 (ATCC# HTB-22), and 8988T cells were grown in DMEM (Lonza) supplemented with 15% Fetal Calf Serum. For gene knockouts, commercially available CRISPR/Cas9 KO plasmids were used (Santa Cruz Biotechnology sc-404890 for HUWE1, sc-406099 for RAD18). Single transfected cells were sorted into 96-well plates, and resulting colonies were screened by Western blot. Gene knockout was confirmed by genomic sequencing.

Native whole cell extracts for co-immunoprecipitation and GST-pulldown studies, were obtained by incubating cells for 30 min on ice with HEPES lysis buffer (50 mM HEPES, 1% Triton, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 10 mM MgCl₂ containing protease inhibitors). For co-immunoprecipitation experiments, lysates were prepared in the presence of 20 mM crosslinking agent DSP (Thermo Scientific). Denatured whole cell extracts were prepared by boiling cells in 100 mM Tris, 4% SDS, 0.5 M β -mercaptoethanol.

In vitro ubiquitination was performed as previously described for HUWE1 (Chen et al., 2005), using commercially available recombinant UBE1 E1 and UBCH7 E2 (Boston Biochem).

Antibodies used in this study were: HUWE1 (Novus NB100-652 and Bethyl A300-486A), PCNA (Abcam ab29), BrdU and IdU (BD 347580), CldU (Abcam ab6326), Tubulin (Genetex gt114), Rad18 (Novus NB100-61063), c-Myc (Santa Cruz Biotechnology sc-40), Actin (Genetex gt5512), γ H2AX (Santa Cruz Biotechnology sc-101696), H2AX (Novus NB100-638), p-RPA (Bethyl A300-245A), RNF168 (Millipore 06-1130), RNF2 (Abcam ab101273), BMI1 (Abcam ab126783), BRCA1 (Santa Cruz Biotechnology sc-642), BRCA2 (Calbiochem OP95), 53BP1 (Bethyl A300-272A), p-Chk1 (S317) (Cell Signaling Technology 2344S), p-Chk2 (T68) (Cell

Signaling Technology 2661S), TOPBP1 (Novus NB100-217), and p-p53 (S15) (Cell Signaling Technology 9284P).

2.2. IMMUNOFLUORESCENCE

Cells were first incubated in pre-extraction buffer (25mM HEPES pH7.5, 50mM NaCl, 1mM EDTA, 3mM MgCl₂, 300mM glucose, 0.5% Triton X-100) for 5 minutes on ice, and then fixed in 4% formaldehyde for 10 minutes at room temperature. Cells were permeabilized with 0.2% Triton X-100 for 5 minutes at room temperature. Slides were blocked with 2% BSA in PBS with 0.2% Tween, and incubated with primary antibodies (diluted in blocking buffer) overnight at 4°C. Next, slides were washed three times in blocking buffer and incubated with secondary antibodies Alexa Fluor 568 and 488 (Invitrogen A11036 and A11001) for 1 hour at room temperature.

2.3. PLASMIDS AND siRNA

The cDNA for HUWE1^{C-ter} (3875-end) was obtained by gene synthesis (GeneArt, Invitrogen). The cDNA for full-length Myc-tagged HUWE1 was purchased from Origene (Cat. No. RC215250). The cDNA for H2AX was obtained from PlasmID (clone ID HsCD00415019). For transient transfection, cDNA fragments were cloned into pCMV-Myc (Clontech) with a GFP tag. For bacteria expression, HUWE1^{C-ter} was cloned into pGEX-6P1 (GE Healthcare). Stable cell lines were obtained by selection with 10mg/ml Geneticin. Plasmid and siRNA transfections were performed using Lipofectamine LTX and Lipofectamine RNAiMAX (Invitrogen), respectively. For gene knockdown, cells were transfected with siRNA twice on consecutive days. The siRNA targeting sequences used are as follows:

siHUWE1#1: CATGAGACATCAGCCCACCCTTAAAA

siHUWE1#2: CACACCAGCAATGGCTGCCAGAATT (unless indicated otherwise in the figures, this was the targeting sequence used for HUWE1)

siHUWE1 5'UTR: AGCCTGACCTGAGTGGGTTAGTGAT

siRNF2 #1: GAGGCAATAACAGATGGCTTAGAAA

siRNF2 #2: TCCAGTAATGGATGGTGCTAGTGAA

siBMI1: AATGGAAGTGGACCATTCCTTCTCC

siRNF168: ACAGGACAGGTTATTGGCATTACAA

siH2AX #1: CGCGACAACAAGAAGACGCGAATCA

siH2AX #2: GCGACAACAAGAAGACGCGAATCAT

2.4. BRDU/PI BI-DIMENSIONAL FLOW CYTOMETRY

As previously described (Fu et al., 2013; Nicolae et al., 2014), cells were incubated with 20 μ M BrdU for 30 min and then harvested by trypsinization and fixed in 70% ethanol overnight. Cells were incubated in 0.1 M HCl/0.5% Triton X-100 for 10 min on ice. Samples were spun down, resuspended in water, boiled for 10 min, and placed on ice for 10 min. Samples were then incubated with primary antibody (Anti-BrdU, BD 347580) and subsequently with secondary antibody Alexa Fluor 488 (Invitrogen A11001) for 30 min each. Before flow cytometry analysis, cells were resuspended in PBS containing 20 μ g/ml RNase and 5 μ g/ml propidium iodide.

2.5. DNA FIBER ASSAY

Cells were incubated with 20 μ M CldU for 30 min. Cells were washed with PBS (and irradiated with 30 J/m² UV-B if indicated). Fresh media containing 20 μ M IdU were added for another 30 min. Cells were lysed in 0.5% SDS, 200 mM Tris-HCl pH 7.4, and 50 mM EDTA. Slides were fixed with methanol:acetic acid (3:1) for 5 min, washed with 2.4 N HCl, and blocked

in 5% BSA in PBS. Slides were incubated with primary antibodies (Abcam 6326 for detecting CIdU; BD 347580 for detecting IdU), washed three times with PBS, incubated with secondary antibodies Alexa Fluor 568 and 488 (Invitrogen A11031 and A21208), washed three times with PBS, and mounted.

2.6. ALKALINE COMET ASSAY

The alkaline comet assay was performed using the CometAssay Kit (Trevigen 4250) according to manufacturer's instructions.

2.7. CLONOGENIC ASSAY

For clonogenic assays, cells were transfected with siRNA for 48 hours then seeded at low densities in 6-well plates and allowed to form colonies. The cells were fixed in 10% methanol/10% acetic acid and stained with 1% crystal violet.

2.8. CHROMATIN IMMUNOPRECIPITATION FOR FRA3B

Cells were cross-linked with formaldehyde (1% final concentration), quenched with glycine (125 mM final concentration) and lysed in 1% SDS, 10-mM EDTA, 50-mM Tris-HCl pH 8. Following sonication, the lysate was diluted 10-fold in Tris-HCl pH 8 buffer, and Triton X-100 was added to 1% final concentration. Extracts were incubated overnight with 5 µg of PARP14 or control mouse IgG, and Protein A/G Sepharose (Santa Cruz Biotechnology). The beads were washed in 10-fold diluted lysis buffer and eluted in 1% SDS, 0.1 M NaHCO₃. Eluates were treated with RNase and Proteinase K, and DNA was purified with a PCR purification kit (Omega). Resultant DNA was dissolved in water and subjected to qRT-PCR using FRA3B (for: ACGTGTGAATGAGGCAGAGA; rev: GTTTGTGGGGTGCTATGCTT), FRA7H (for:

TAATGCGTCCCCTTGTGACT; rev: GGCAGATTTTAGTCCCTCAGC) and GAPDH (for: TGCACCACCAACTGCTTAGC; rev: TCAGCTCAGGGATGACCTTG).

2.9. iPOND

Cells (approx. 10^8 for each condition) were incubated with 10 μ M EdU (5-ethynil-2'-deoxyuridine; Invitrogen) for 20 min, fixed in 1% formaldehyde for 25 min, and scraped in PBS with 0.01% Triton X-100. Cells were then permeabilized in PBS with 0.25% Triton X-100 for 20 min and incubated in click reaction buffer (10 mM sodium ascorbate, 2 mM CuSO₄, 10 μ M azide-PEG11-biotin, 0.01% Triton X-100, 0.5% BSA, in PBS) for 90 min at room temperature. Next, cell pellets were resuspended in RIPA buffer, sonicated, and cleared by high-speed centrifugation. Nascent DNA was captured by incubation of the cleared extracts with Streptavidin T1 Dynabeads (Invitrogen) for 60 min. The beads were then boiled for 30 min in Laemmli buffer containing 100 mM DTT to elute the proteins and reverse the cross-links.

2.10. RNA SEQUENCING

Total RNA was extracted using Trizol (Invitrogen). RNA integrity number (RIN) was measured using a BioAnalyzer (Agilent) RNA 6000 Nano Kit to confirm RIN above 7. The cDNA libraries were prepared using the SureSelect Strand Specific RNA Library Preparation Kit (Agilent) per the manufacturer's instructions. The final product was assessed for size distribution and concentration using BioAnalyzer High Sensitivity DNA Kit (Agilent). Pooled libraries were denatured and loaded onto a TruSeq Rapid flow cell on an Illumina HiSeq 2500 and run for 50 cycles using a single-read recipe according to the manufacturer's instructions. De-multiplexed sequencing reads which passed the default purify filtering of Illumina CASAVA pipeline (version 1.8) were quality trimmed/filtered using the FASTX-Toolkit

(http://hannonlab.cshl.edu/fastx_toolkit) by having a quality score cutoff of 20. The filtered reads were aligned to human reference genome (hg38) using TopHat (v2.0.9) by allowing up to 2 mismatches. Differential gene expression was calculated using Cufflinks v2.0.2 provided with the Ensembl gene annotation (release 78). A corrected $p < 0.05$ was applied to determine significant differences. Ingenuity Pathway Analysis (IPA, www.qiagen.com/ingenuity) was used to identify functionally relevant signaling pathways in the differentially expressed gene cluster(s).

2.11. STATISTICAL ANALYSES

For all functional assays, the statistical analysis performed was the *t*-test (two-tailed, equal variance), using PRISM software. Statistical significance is indicated for each graph (ns = not significant, for $p > 0.05$; * for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$). The exact p-values are indicated in the figure legends. All Western blots, co-immunoprecipitations, and FACS analyses are representative of at least three independent experiments.

Chapter 3. RESULTS

3.1. HUWE1 IS REQUIRED FOR DNA DAMAGE TOLERANCE AND MAINTENANCE OF GENOMIC STABILITY

A broad range of substrates have been identified for HUWE1-mediated ubiquitination. However, mechanistic understanding of the pathways controlled by HUWE1 is still lacking. To address this, we employed the CRISPR/Cas9 technology to knock out HUWE1 in human embryonic kidney 293T cells, HeLa cervical adenocarcinoma cells, and 8988T pancreatic adenocarcinoma cells (Fig 6A-C).

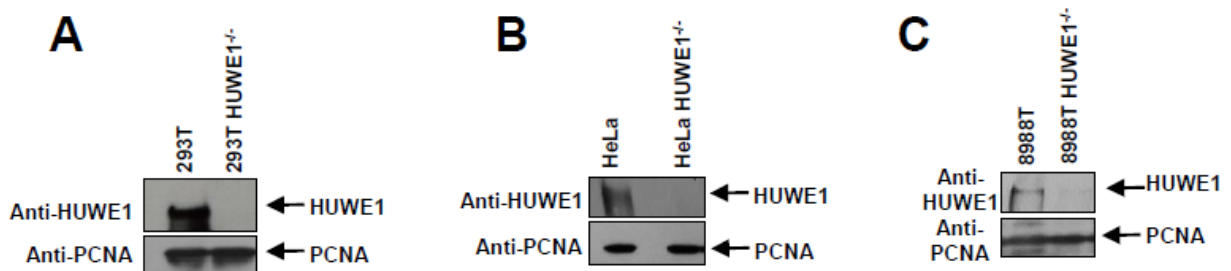


Figure 6. HUWE1-knockout cells. Western blot showing the absence of HUWE1 protein in 293T (A), HeLa (B), and 8988T (C) cells subjected to CRISPR/Cas9-mediated HUWE1 deletion.

We subjected 293T HUWE1-knockout cells to RNA-sequencing. Examination of the global gene expression profile using Ingenuity Pathway Analysis (Fig 7) showed dysregulation of cancer-related processes (nine out of the top ten hits). This indicates a broad impact of HUWE1 on the cellular transformation process.

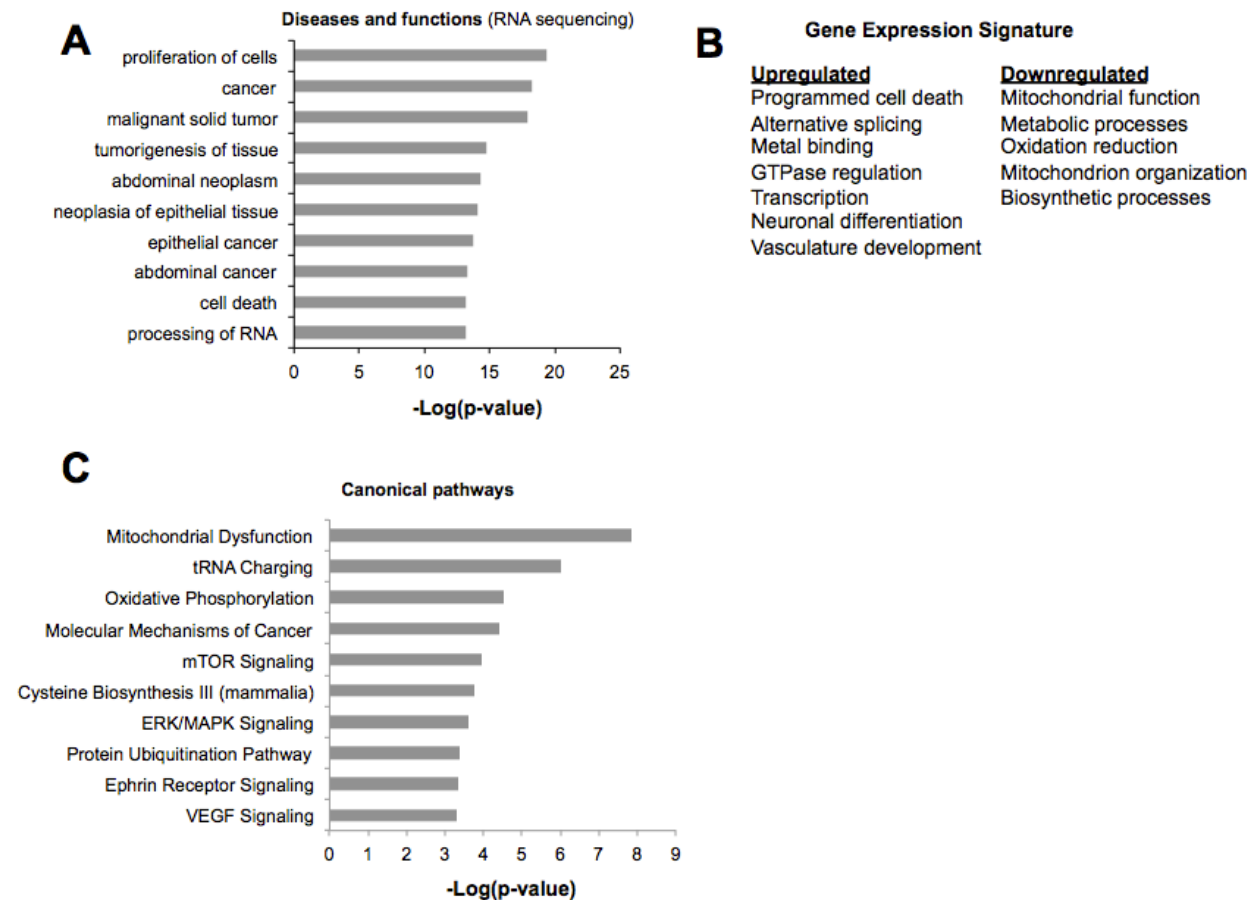
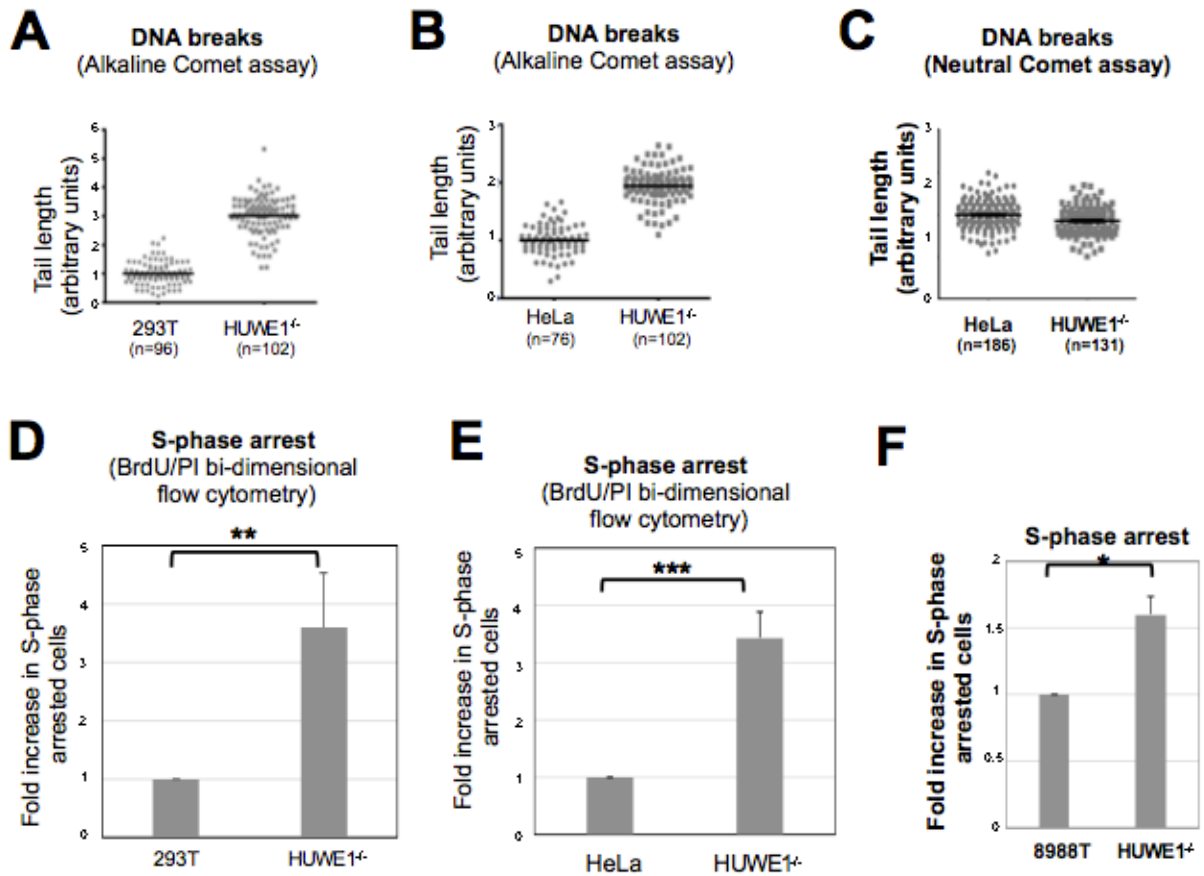


FIGURE 7. Gene expression profile in HUWE1-knockout cells. Pathway analyses showing the processes dysregulated in HUWE1-knockout cells compared to control 293T cells. A. Ingenuity pathway analyses of RNA sequencing experiments, showing the top ten items in the category Diseases and functions, that are dysregulated in HUWE1-knockout 293T cells compared to control cells. B. RNA sequencing data was analyzed using DAVID. C. Ingenuity pathway analysis of the RNA sequencing data, showing the top ten hits in the category Canonical pathways. Triplicate experiments were performed for each cell line.

While HUWE1 has been shown to regulate a number of tumor suppressors and oncogenes, our RNA-seq study in 293T cells suggested a more direct effect of HUWE1 on cellular integrity. Thus, we investigated if HUWE1 loss correlates with genomic instability, a trait that enables cellular transformation and cancer. Strikingly, HUWE1-knockout cells showed a significant increase in DNA breaks in the absence of any DNA damage treatment, as measured by the alkaline comet assay (Fig 8A-C). These findings suggest that there is increased replication stress in the absence of HUWE1. Indeed, cell cycle distribution analyses using BrdU/PI bi-dimensional flow cytometry indicated increased S-phase arrest (cells with S-phase DNA content, but negative for

BrdU incorporation), coupled with a reduction in BrdU-positive cells undergoing DNA synthesis (Fig 8D-I). Moreover, the DNA fiber assay showed that HUWE1-knockout cells have shorter replication tracts (Fig 8J and K), indicative of replication stress. Finally, we also employed siRNA (Fig 9A-C) to transiently downregulate HUWE1 in 293T, 8988T, and HeLa cells. Similar to the knockout cells, HUWE1-knockdown cells showed increased S-phase arrest, a smaller proportion of BrdU-positive cells undergoing DNA synthesis, and reduced replication tract length (Fig 9D-I). These data indicate that HUWE1-deficient cells are unable to resolve endogenous DNA damage, resulting in DNA replication dysfunction.



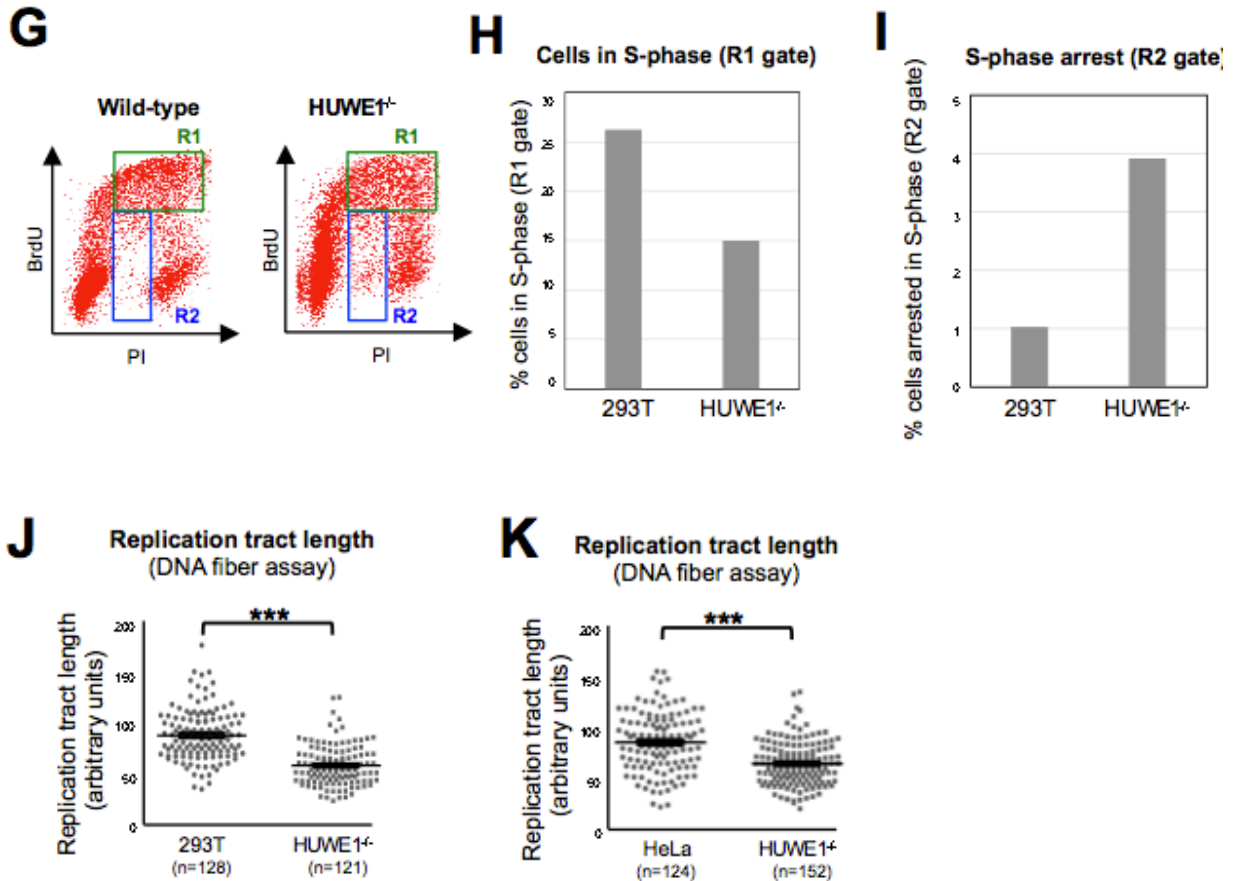


Figure 8. HUWE1-knockout cells show genomic instability and increased replication stress. A, B. HUWE1-knockout 293T (A) and HeLa (B) cells show increased DNA breaks in the absence of exogenous DNA damage treatment. Results from the Alkaline Comet Assay are shown. The “n” numbers of comet tails analyzed (pooled from two independent experiments), as well as the mean, are indicated on the graphs. HUWE1-knockout HeLa cells did not show increased breakage in the Neutral Comet assay (C), indicating that the majority of breaks observed in cycling HUWE1-knockout cells are not double strand breaks, but rather single strand breaks and other types of lesions. D, E. Increased S-phase arrest in HUWE1-knockout 293T (D) and HeLa (E) cells. Cycling cells were incubated with BrdU and subjected to BrdU/PI bi-dimensional flow cytometry. Representative flow cytometry profiles are presented in Fig G-I. Bars represent the fold increase in the percentage of cells with S-phase DNA content (between 2N and 4N) but negative for BrdU staining. Bars represent the average of three independent experiments, with error bars showing SD. The p-values are 0.0091 (D) and 0.0007 (E). J, K. The DNA fiber assay shows reduced replication tract length in HUWE1-knockout 293T (J) and HeLa (K) cells in the absence of exogenous DNA damage treatment. The “n” numbers of fibers analyzed (pooled from three independent experiments), as well as the mean +/- SEM, are indicated on the graphs. P-values are 9.8×10^{-22} (J) and 1.0×10^{-10} (K).

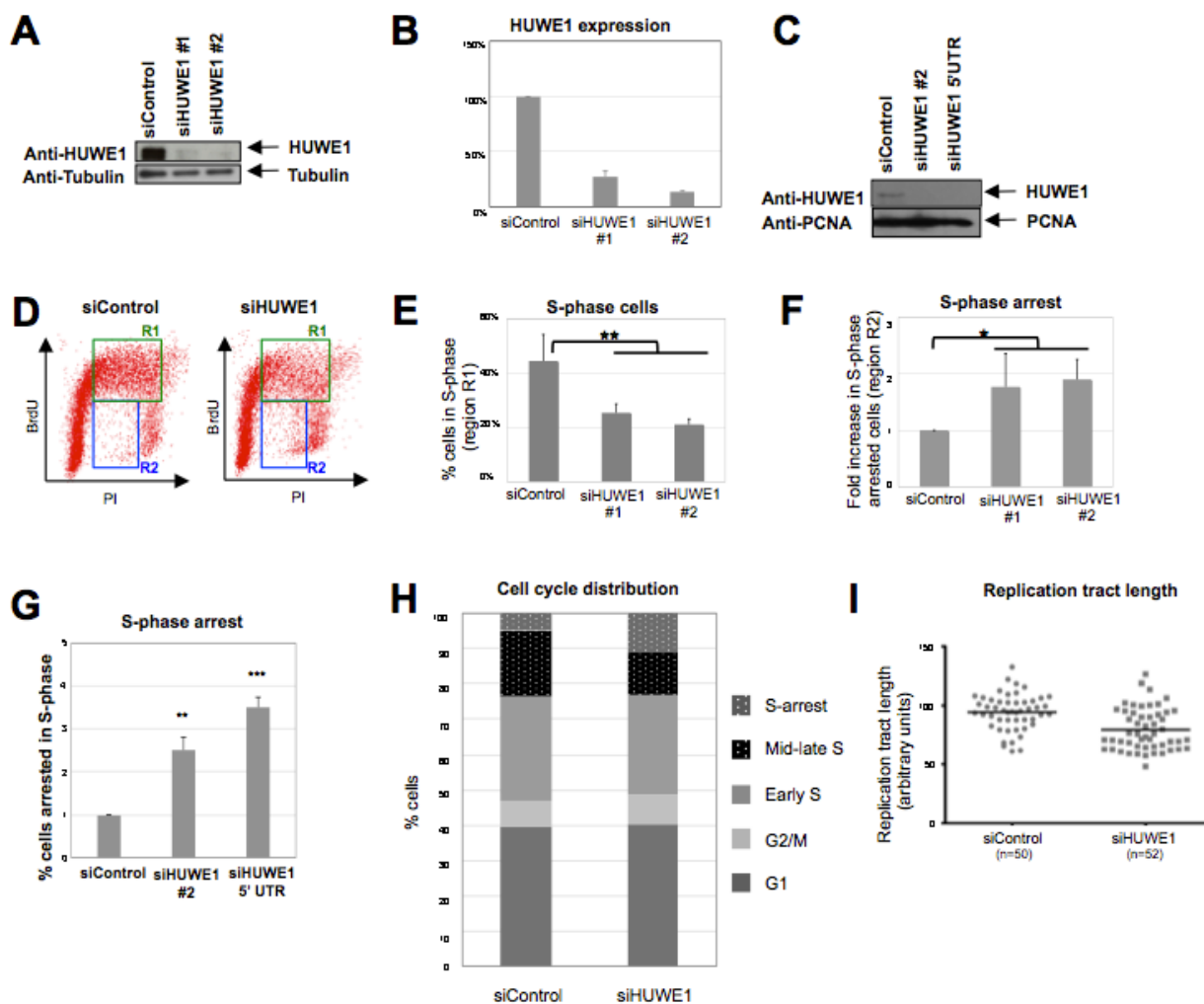


Figure 9. Increased replication stress in HUWE1-knockdown cells. A. HUWE1 levels are efficiently downregulated by two different HUWE1 siRNA oligonucleotides in 8988T cells. B. Quantification of HUWE1 expression following siRNA treatment. Band intensity was quantified using ImageJ software and normalized to Tubulin loading control. The average of three experiments is shown. Error bars indicate SD. C. Western blot showing that the siRNA oligonucleotides used are efficiently knocking down HUWE1 levels in 293T cells. D-F. Cell cycle analyses by flow cytometry using BrdU/PI double staining show increased replication arrest in HUWE1-depleted 8988T cells in the absence of exogenous DNA damage treatment. D. Representative flow cytometry profiles of control and HUWE1-knockdown cells. R1-labeled region indicates mid and late S-phase cells (BrdU-positive, >2N DNA content), while R2-labeled region indicates S-phase arrested cells (BrdU-negative, DNA content between 2N and 4N). E. Quantification of S-phase cells. Percentage of cells in R1 region is shown. Bars represent the average of three independent experiments. Error bars indicate SD. P-value is 0.0014. F. Quantification of S-phase arrested cells. Bars represent the fold increase in the percentage of cells in R2 region, normalized to siControl-treated cells. The average of three independent experiments is shown. Error bars indicate SD. P-value is 0.0135. G. Increased S-phase arrest in 293T cells following HUWE1 knockdown. The percentage of cells arrested in S-phase is shown. The average of five experiments, with standard deviations, is presented. The P-values shown (calculated using the t-test two-tailed, equal variance) indicate the statistical significance relative to siControl (0.0024 and 0.0001, respectively). H. Quantification of cell cycle distribution of control and HUWE1-depleted 293T cells, showing the altered distribution of mid-late S-phase vs. S-phase-arrested cells. Shown are G1 (BrdU-negative, 2N DNA content); G2/M (BrdU-negative, 4N DNA content); early S (BrdU-positive, 2N DNA content); mid-late S (BrdU-positive, >2N DNA content); S-arrest (BrdU-negative, DNA content between 2N and 4N). The quantification of one representative plot is shown, but this result was reproduced in many independent experiments. I. DNA fiber experiment showing reduced replication tract length in HUWE1-depleted HeLa cells in the absence of exogenous DNA damage treatment. The “n” numbers of fibers analyzed (pooled from two independent experiments), as well as the mean, are indicated on the graphs.

This novel activity of HUWE1 in protecting the replication fork against DNA damage was rather unexpected, since previous studies described HUWE1 as a negative regulator of DNA repair through suppression of HR and BER (Khoronenkova and Dianov, 2011; Markkanen et al., 2012; Parsons et al., 2009; Wang et al., 2014). Therefore, we analyzed the impact of HUWE1 on the cellular sensitivity to hydroxyurea and UV radiation, agents that induce replication fork stalling during S-phase. Clonogenic experiments indicated that HUWE1-knockdown and knockout cells are hypersensitive to these agents (Fig 10A-D). Moreover, DNA fiber assays showed an even stronger reduction of replication tract length in HUWE1-knockdown cells exposed to UV (Fig 10F and G). Altogether, these results indicate a novel role for HUWE1 in DNA damage tolerance.

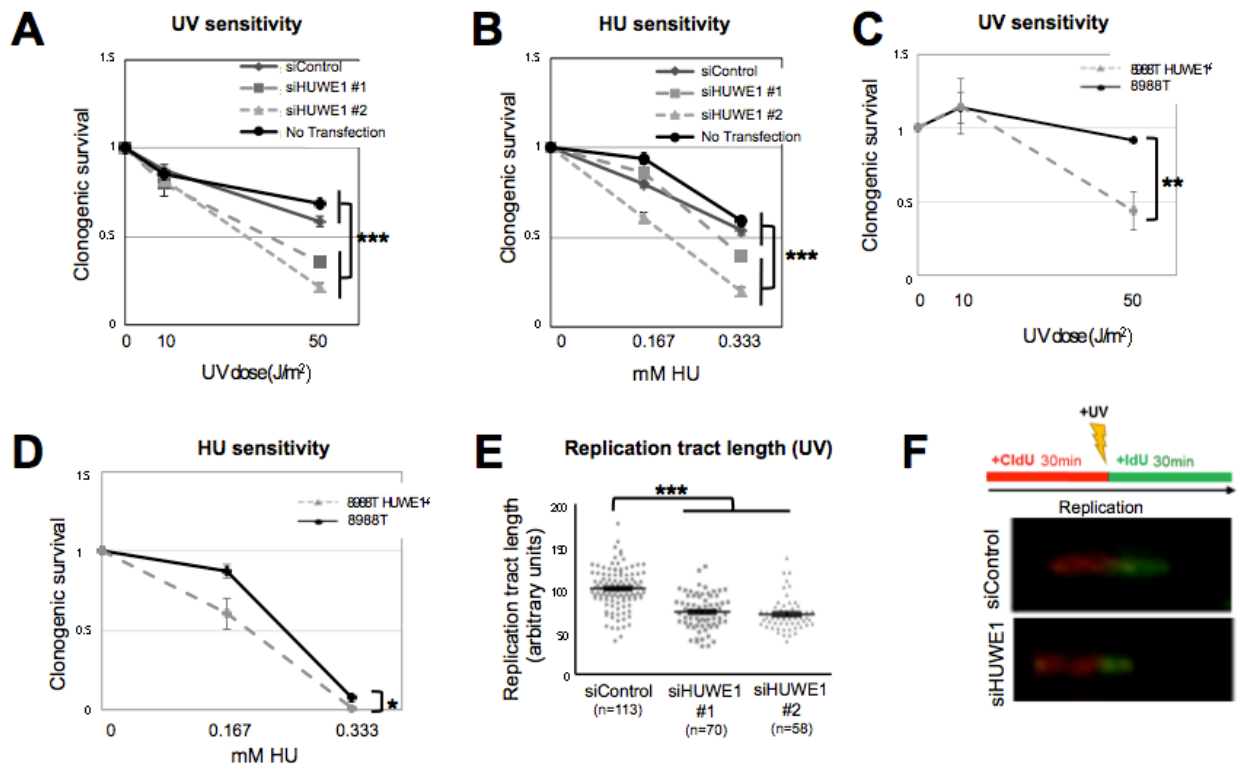


Figure 10. HUWE1-knockdown and knockout cells are hypersensitive to replication fork-stalling agents. A, B. Clonogenic assay showing that HUWE1 knockdown in 8988T cells are sensitive to UV (A), and HU (B). As controls, both cells transfected with non-targeting siRNA (siControl), and non-transfected cells, were used. Note that the siHUWE1#2 oligonucleotide shows a stronger downregulation of HUWE1, and confers increased sensitivity compared to siHUWE1#1. The average of nine experiments is shown. Error bars represent SEM. P-values (representing the statistical difference between the samples at the highest dose treatment) are: 4.55×10^{-9} (A) and

1.8x10⁻⁸ (B). C, D. UV (C) and HU (D) sensitivity of HUWE1-knockout 8988T cells. The average of three experiments, with standard deviations, is shown. P-values (calculated using the t-test two-tailed, equal variance) are 0.0029 (C) and 0.0126 (D). E. DNA fiber experiments showing decreased replication tract length following UV-B treatment (30J/m²) in HUWE1-depleted HeLa cells. The “n” numbers of fibers analyzed (pooled from three independent experiments), as well as the mean +/- SEM, are indicated on the graphs. P-value is 2.11x10⁻²⁰. F. Schematic of the experimental setup, including examples of micrographs, for the DNA fiber experiment.

3.2. HUWE1 INTERACTS WITH THE REPLICATION FACTOR PCNA AT STALLED REPLICATION FORKS

We next investigated if HUWE1 is directly coupled to the replication machinery. Using the iPOND (Isolation of Proteins On Nascent DNA) technique that allows identification of proteins at replication forks (Dungrawala and Cortez, 2015; Sirbu et al., 2011) we found that HUWE1 is detectable in unchallenged replisomes, and accumulates after UV exposure (Fig 11A). Immunofluorescence experiments confirmed that HUWE1 localizes to chromatin foci in response to replication fork stalling induced by HU or UV (Fig 11B).

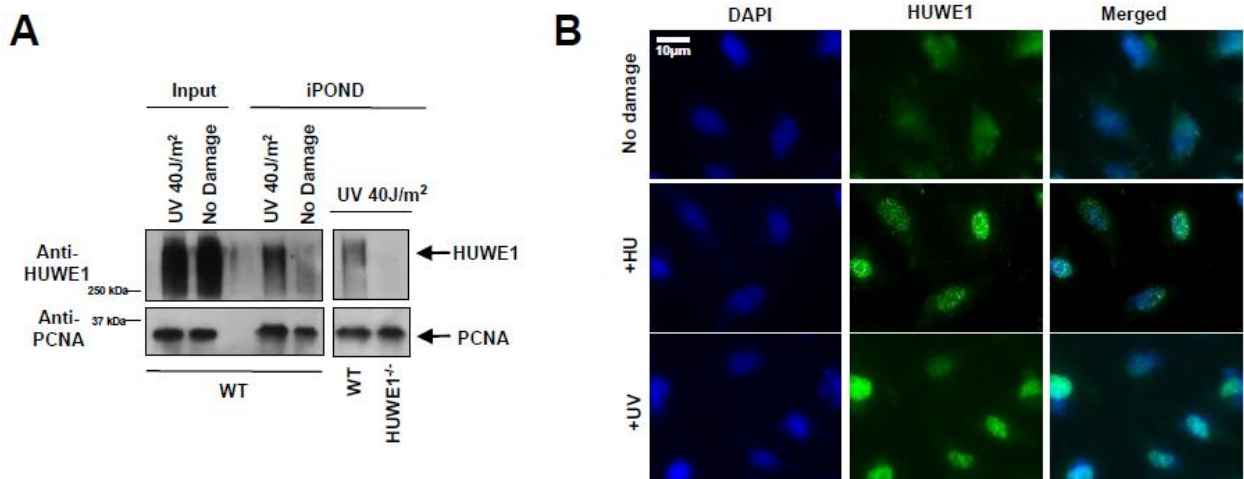


Figure 11. HUWE1 localizes to replication forks. A. iPOND experiment showing that HUWE1 localizes to replication forks in 293T cells. Binding to nascent DNA increased after UV exposure. Because of the difficulties in removing all the crosslinks in high molecular weight proteins, HUWE1 is detected as a high molecular weight smear. A control experiment using the HUWE1-knockout cells (shown in the right side panel) confirmed the specificity of the HUWE1 signal. B. Immunofluorescence experiment showing that HUWE1 localizes to chromatin foci in HeLa cells exposed to replication fork stalling agents HU (2mM for 16h) and UV (40J/m², analyzed 2h later).

Importantly, these foci show a high degree of overlap with PCNA foci, an essential replication fork component that regulates the restart of stalled replication forks (Fig 12).

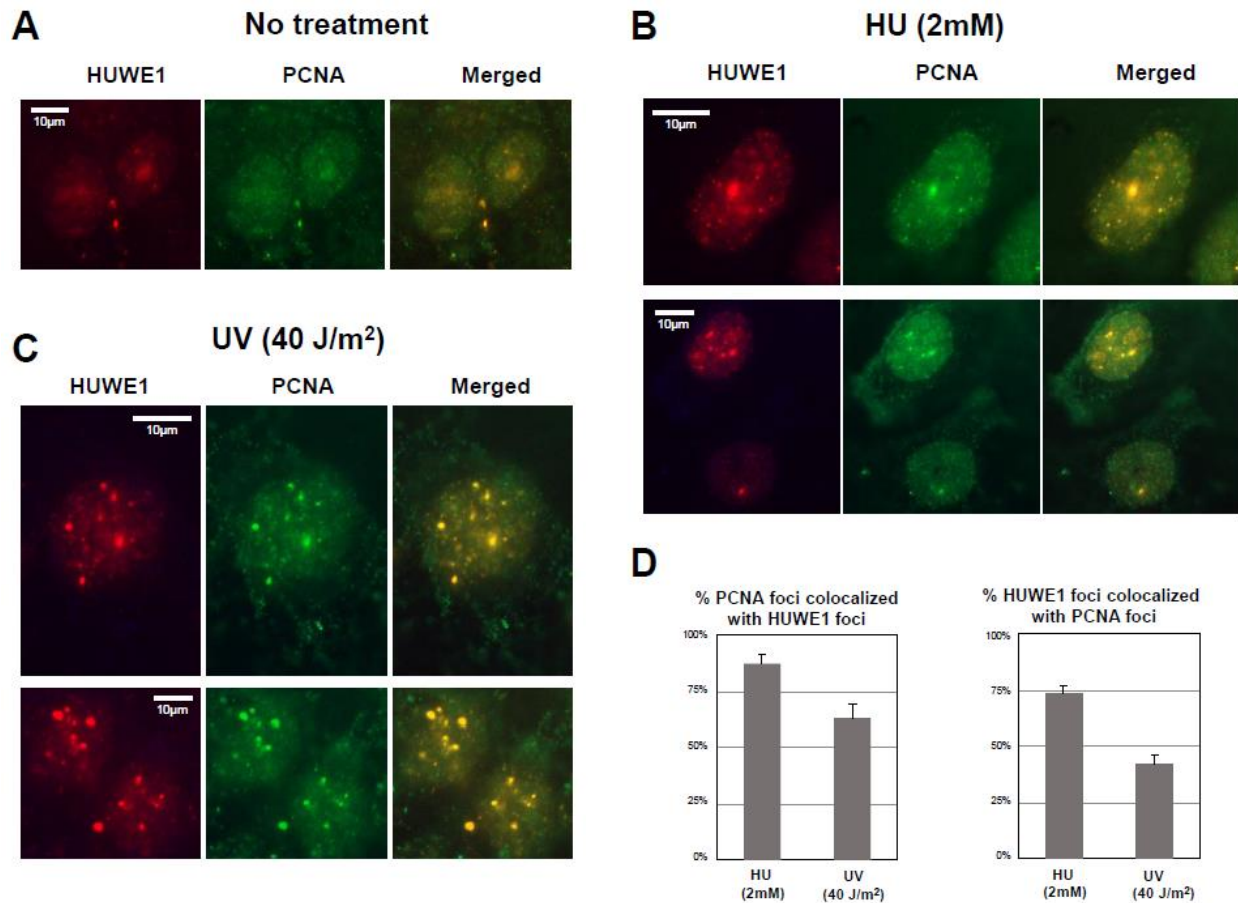


Figure 12. Co-immunofluorescence experiments showing co-localization of HUWE1 and PCNA. A–C Representative immunofluorescence micrographs of U2OS cells untreated (A), treated with 2 mM HU for 24 h (B), or exposed to 40 J/m² UV and analyzed 2 h later (C). D. Quantifications showing the percentage of PCNA foci co-localizing with HUWE1 foci and, reciprocally, the percentage of HUWE1 foci co-localizing with PCNA foci. Only cells with more than five foci were analyzed. The average of three experiments, with standard errors, is shown. In total, 191 HUWE1 and 156 PCNA foci were counted.

This co-localization suggested that HUWE1 might directly interact with PCNA. HUWE1 is a very long polypeptide (4374 amino acids), containing a HECT-type ubiquitin ligase domain in its C-terminus. Analysis of its amino acid sequence identified a putative PCNA interacting motif (PIP-box) **QPAVEAFF** right before the start of the HECT domain (Fig 13).

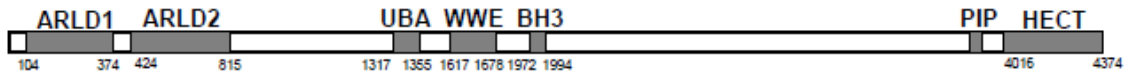


Figure 13. Schematic representation of full-length HUWE1. Shown is the PIP-box domain we describe here (aa3880-3887). ARLD: Armadillo repeat-like domain; UBA: Ubiquitin-associated domain; BH3: Bcl2-homology 3 domain; HECT: Homologous to the E6-AP Carboxyl Terminus (catalytic ubiquitin ligase domain).

Reciprocal co-immunoprecipitation studies of endogenous proteins confirmed that HUWE1 and PCNA interact in 293T, 8988T, and MCF7 cells (Fig 14). This interaction was not reduced in RAD18-knockout cells, showing that it does not require PCNA ubiquitination (Fig 15).

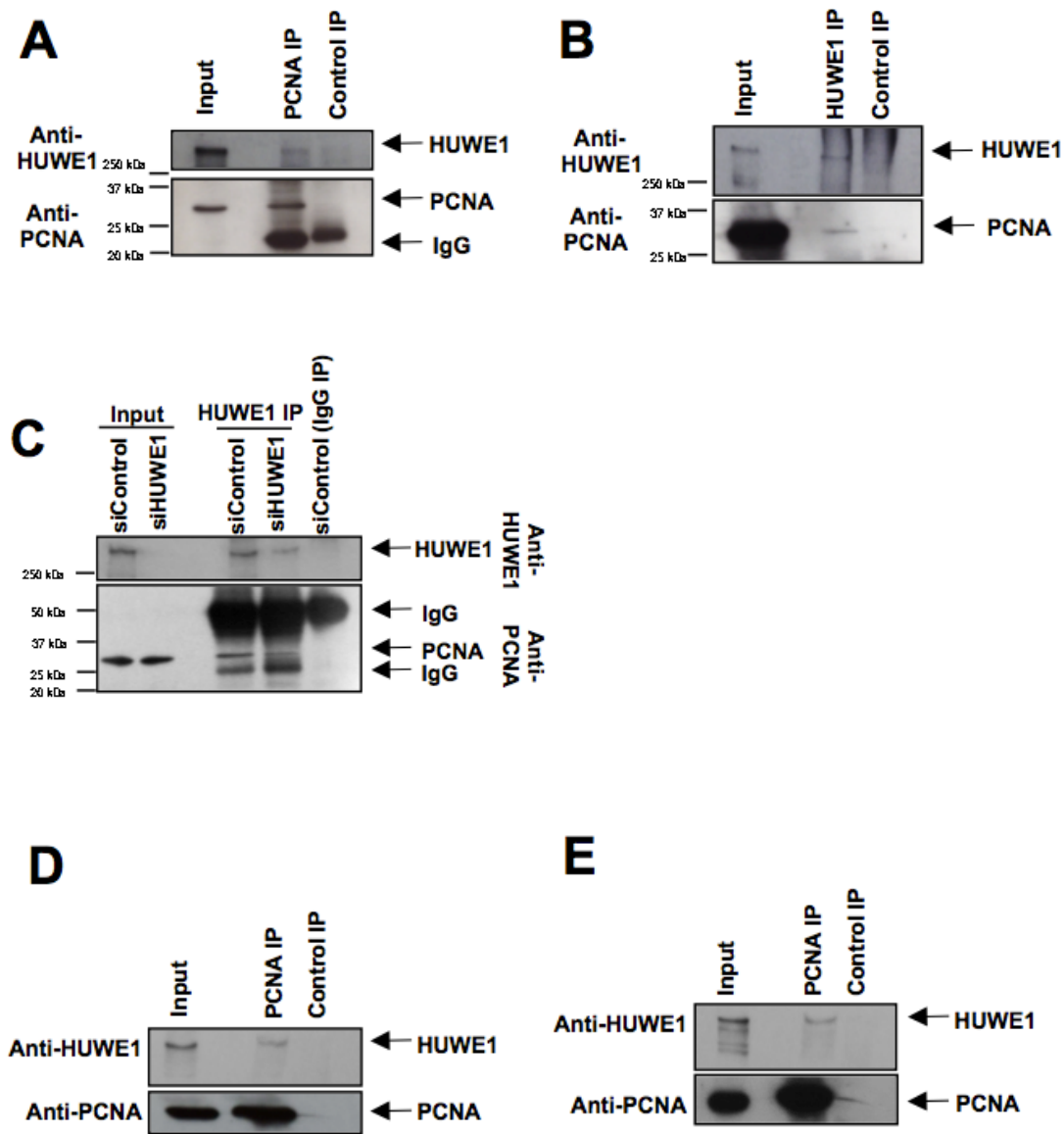


Figure 14. HUWE1 interacts with PCNA. A-C. Interaction between endogenous HUWE1 and PCNA in 293T cells. A. Anti-PCNA immunoprecipitation, showing that HUWE1 co-precipitates. B. Reciprocal experiment showing that PCNA co-precipitates with HUWE1 in anti-HUWE1 immunoprecipitation. C. As control, endogenous HUWE1 was depleted by siRNA treatment, and extracts were subjected to anti-HUWE1 immunoprecipitation. The HUWE1 blot shows that much less HUWE1 is precipitated by anti-HUWE1 antibodies from HUWE1-knockdown cells than control, as expected. PCNA is co-precipitated by anti-HUWE1 antibodies from control, but not HUWE1-depleted cells, showing that the interaction is specific and not due to unspecific binding of PCNA to anti-HUWE1 antibodies. D, E. HUWE1 co-precipitates with PCNA in anti-PCNA immunoprecipitation from extracts of MCF7 (D) and 8988T (E) cells, showing that the HUWE1-PCNA interaction can be detected in different cell lines.

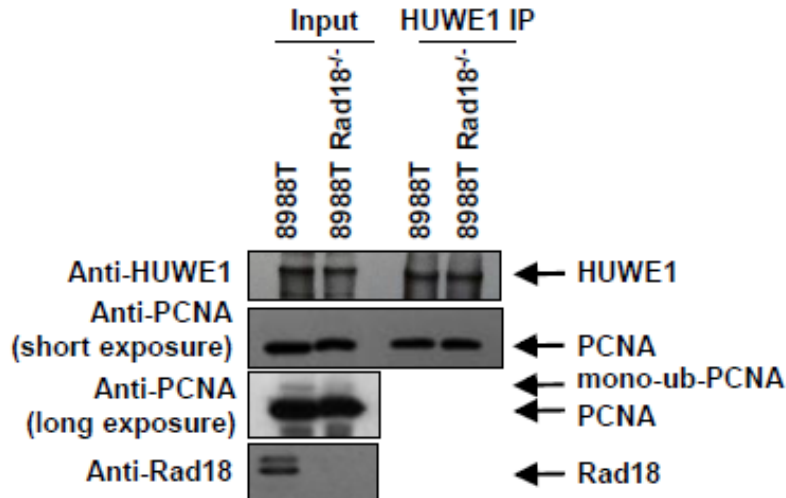


Figure 15. HUWE1-PCNA interaction does not rely on PCNA-ubiquitination. Co-immunoprecipitation experiment from control and RAD18-knockout 293T cells, showing that the HUWE1-PCNA interaction is not affected by loss of PCNA ubiquitination. RAD18-knockout cells were obtained by CRISPR/Cas9 technology. The RAD18 and PCNA blots show loss of RAD18, and of PCNA ubiquitination respectively.

To further analyze the HUWE1-PCNA interaction, we cloned a C-terminal truncation of HUWE1 containing the PIP and HECT domains (HUWE1^{C-ter}; Fig 16A). This truncation strongly interacted with PCNA in co-immunoprecipitation and GST-PCNA pulldown experiments (Fig 16B-D).

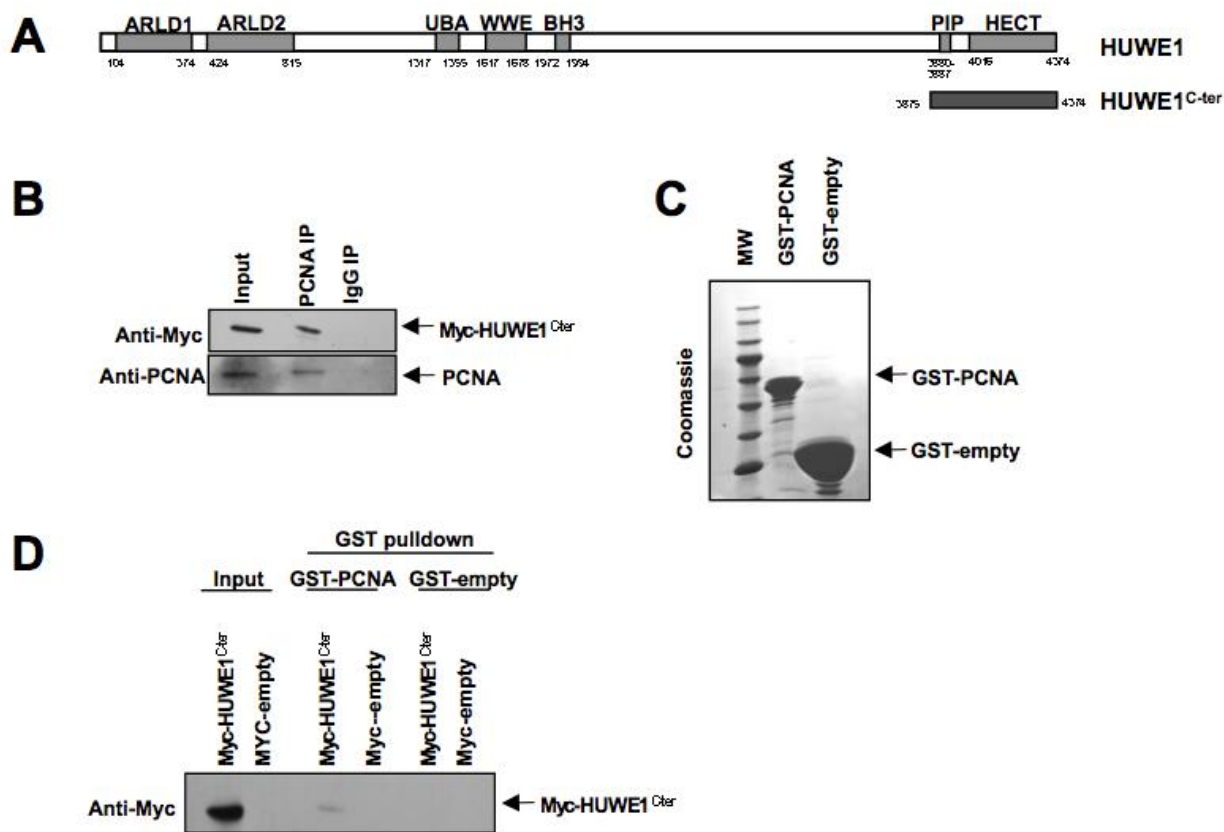


Figure 16. HUWE1^{C-ter} interacts with PCNA. A. The span of the HUWE1^{C-ter} (3875-end) fragment used in subsequent studies is indicated. B. Myc-HUWE1^{C-ter} fragment was transfected in 293T cells. Anti-PCNA immunoprecipitation showed that the C-terminal HUWE1 fragment interacts with PCNA. C. Coomassie staining of recombinant GST-PCNA expressed and purified from bacteria. D. GST-pull-downs using GST-PCNA or GST-empty as control, and extracts of 293T cells transfected with Myc-HUWE1^{C-ter}, showing interaction of this fragment with recombinant PCNA.

To determine the importance of the PIP-box for this interaction, we next introduced point mutations in conserved residues of the PIP motif. These mutations abolished the ability of HUWE1^{C-ter} to interact with PCNA in co-immunoprecipitation and GST-pull-down studies (Fig 17A-D). We also obtained a full-length clone of HUWE1 with a C-terminal Myc epitope tag, and performed Myc-tag immunoprecipitation from cells transiently transfected with wild-type or PIP-box mutant HUWE1. PCNA co-precipitated with full-length wildtype HUWE1, but not the PIP-box mutant variant (Fig 17E). These results show that HUWE1 directly interacts with PCNA through the PIP-box motif.

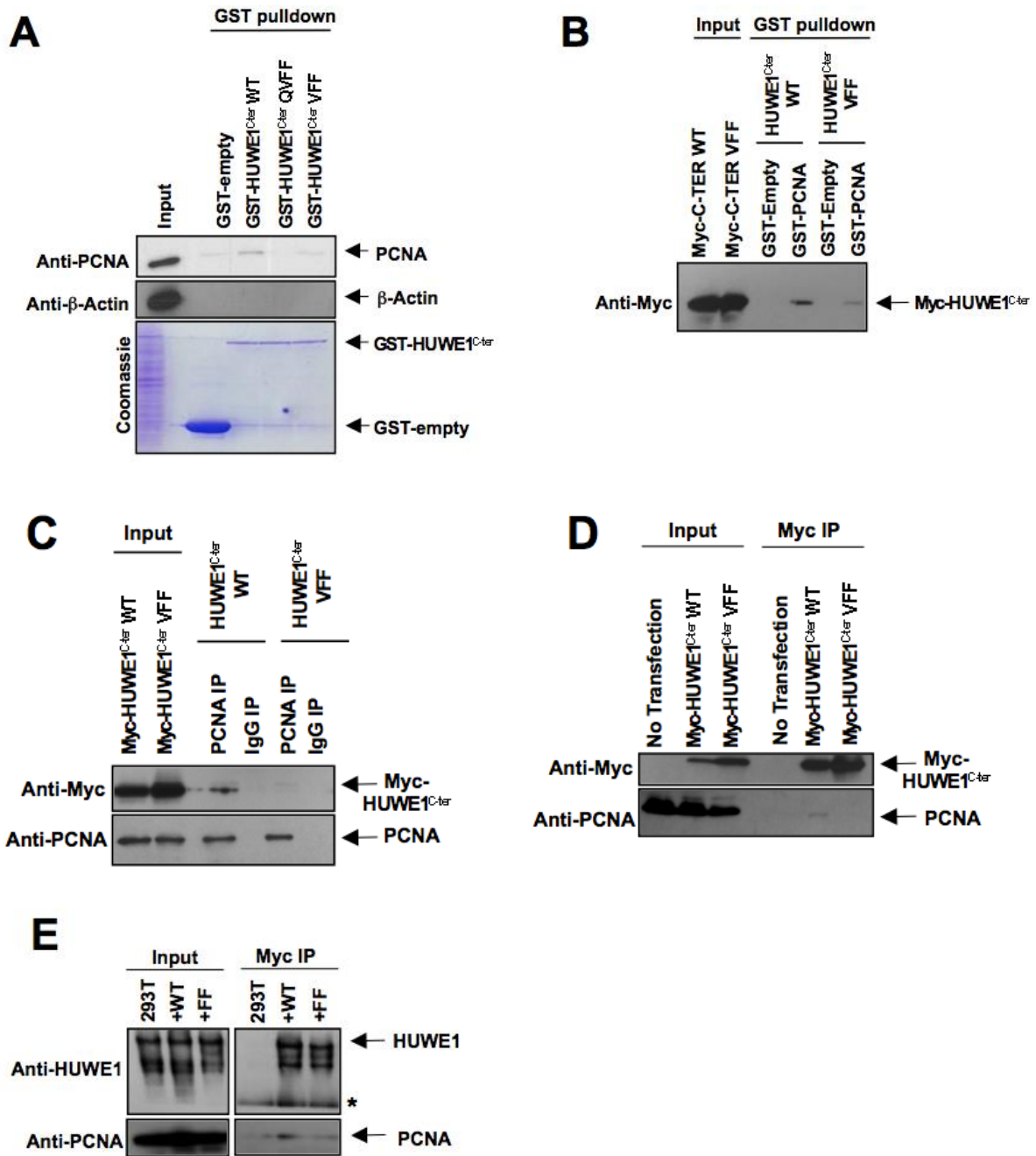


Figure 17. The PIP-box of HUWE1 is essential for its interaction with PCNA. A, B. GST-pull-downs showing that HUWE1 PIP-box mutants do not interact with PCNA. A. Recombinant GST-tagged HUWE1^{C-ter}, either wild-type or PIP-box mutant variants (QVFF and VFF –the indicated residues, critical for the PIP-box, were mutated to A), were purified from bacteria (see Coomassie staining) and employed for GST-pull-downs using extracts of 293T cells. PCNA co-precipitated with wild-type, but not PIP-box mutants. B. Reciprocal GST-pull-down using recombinant GST-PCNA and extracts of 293T cells overexpressing Myc-HUWE1^{C-ter}. Wild-type, but not the PIP-box mutant (VFF) HUWE1 fragment bound to GST-PCNA. C, D. Reciprocal co-immunoprecipitation experiments showing that PIP-box mutation abolishes PCNA interaction. C. 293T cells were transfected with Myc-HUWE1^{C-ter} variants, and subjected to anti-PCNA (C) or anti-Myc (D) immunoprecipitation. Only wild-type, but not PIP-box mutant (VFF) HUWE1 interacted with PCNA in both experimental setups. E. Mutation of the PIP-box in full-length HUWE1 also blocks PCNA interaction. Full-length Myc-tagged HUWE1 (wild-type or PIP-mutant) was transfected in 293T cells. Following Myc-immunoprecipitation, endogenous PCNA was detected in wildtype, but not PIP-box mutant (FF) complexes.

3.3. HUWE1 INTERACTION WITH PCNA IS REQUIRED FOR DNA DAMAGE TOLERANCE

We next set out to investigate the role of HUWE1 interaction with PCNA. For this, we transfected the HUWE1-knockout 293T cells described in Fig 6A with full-length Myc epitope tagged HUWE1. We created cells stably expressing wildtype, PIP-box mutant (FF), or catalytic mutant (C4341A mutation inactivating the HECT domain) HUWE1 variants. HUWE1 Western blots (Fig 18A) showed that expression of the exogenous variants stably transfected in the knockout cells is at a similar level to the expression of endogenous HUWE1 in the parental 293T cell line (with the exception of the HECT mutant, which shows reduced expression).

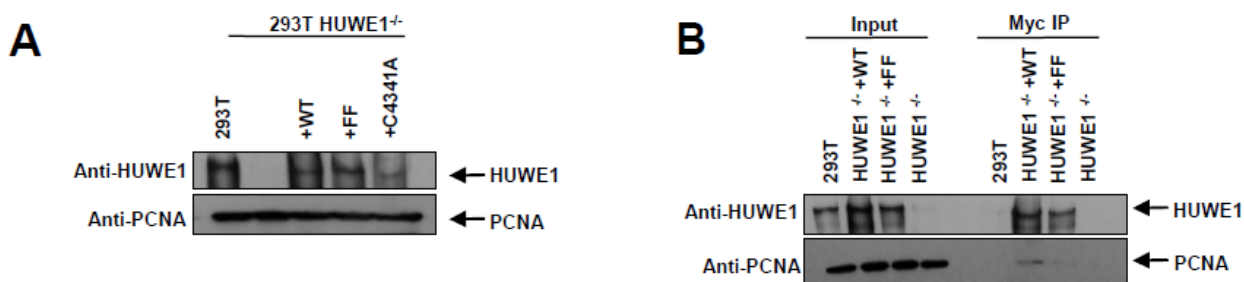


Figure 18. Generation of HUWE1-corrected cells in a knockout background. A. Stable expression of Myc-tagged wild-type, PIP-box mutant, and catalytic inactive HUWE1 variants in HUWE1-knockout 293T cells. The Western blot shows that expression of the Myc-tagged variants stably transfected in the knockout cell lines, is similar to the endogenous HUWE1 expression. B. Myc-tag immunoprecipitation using extracts of the corrected cell lines described above. As expected, PCNA co-precipitates with wild-type, but not PIP-box mutant HUWE1 (FF).

As expected, also in this setup, wild-type, but not the PIP-box mutant, HUWE1 interacts with PCNA as shown by Myc-tag immunoprecipitation experiments (Fig 18B)-further confirming that the HUWE1-PCNA interaction occurs through the PIP-box. iPOND and immunofluorescence experiments showed that PIP-box mutant HUWE1 does not efficiently localize to replication forks (Fig 19A and B), indicating that PCNA interaction is required for correct localization of HUWE1.

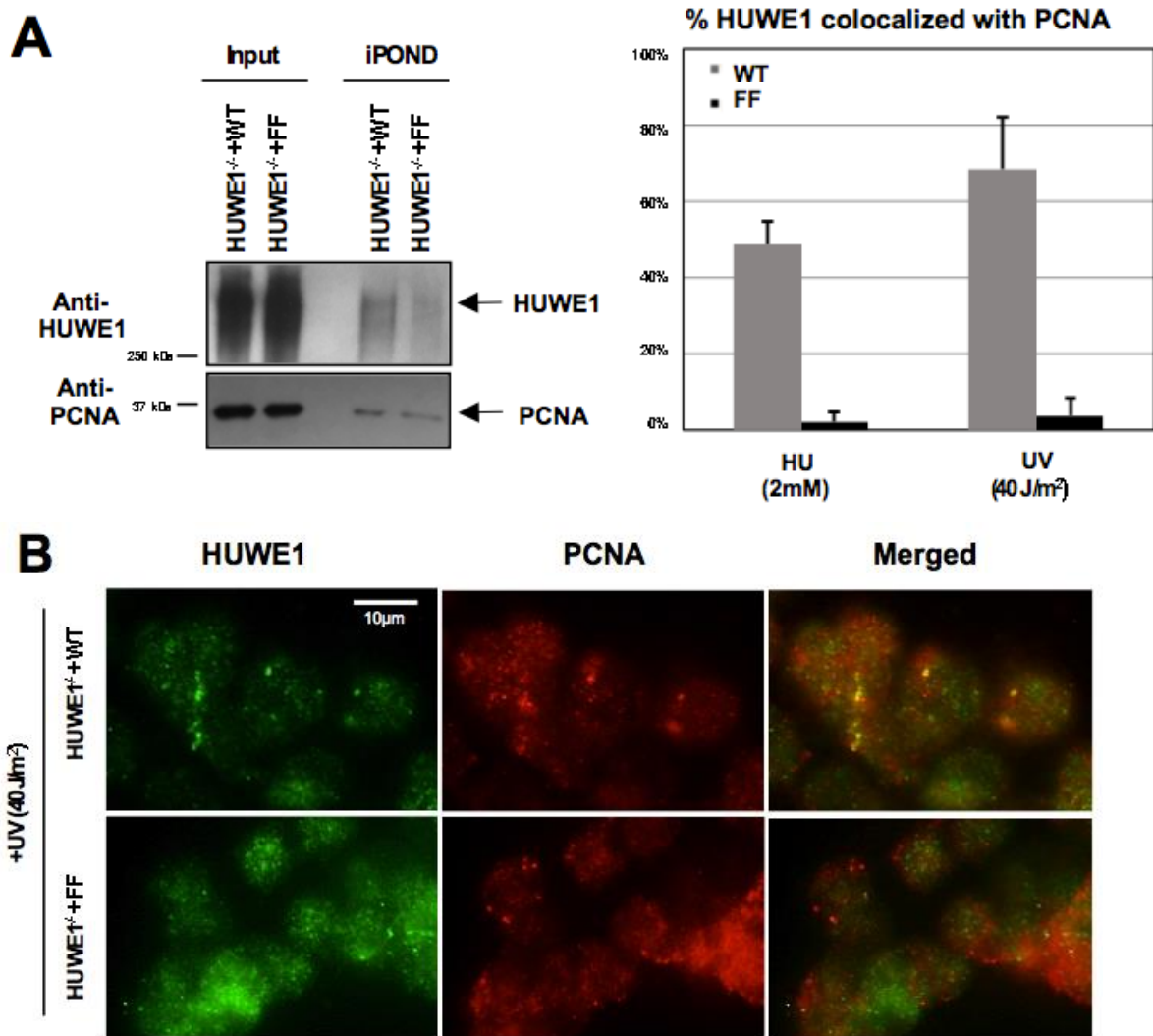
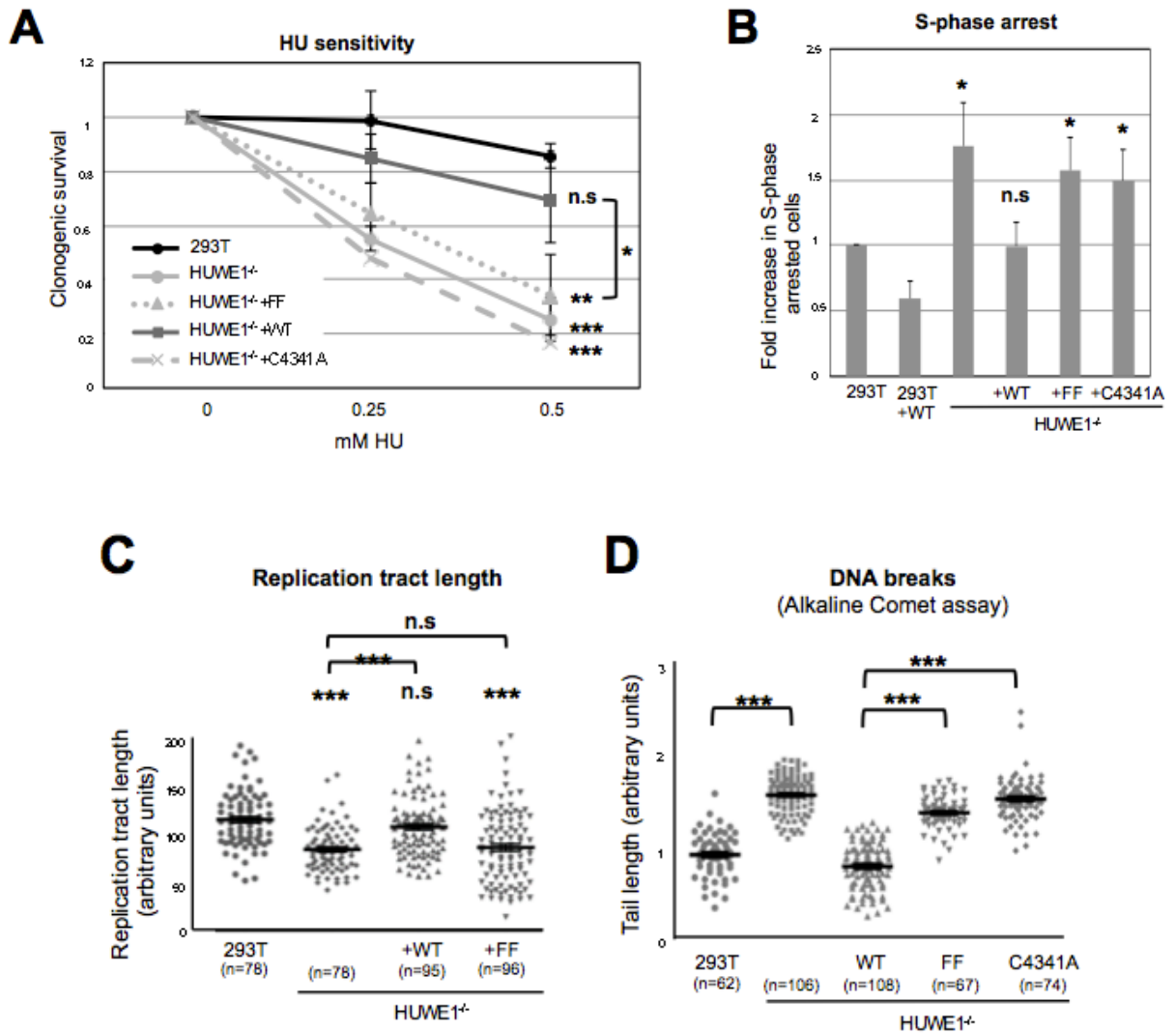


Figure 19. PIP-box mutant HUWE1 does not efficiently localize to replication forks. A. iPOND experiment using HUWE1-knockout 293T cells, corrected with wild-type or PIP-mutant HUWE1. The PIP-mutant shows reduced crosslinking to nascent DNA, indicating defective recruitment to replication forks. B. HUWE1-knockout cells corrected with wild-type or PIP mutant were subjected to immunofluorescence using HUWE1 and PCNA antibodies. Similar to the endogenous protein, Myc-HUWE1 wild-type forms UV- and HU-induced chromatin foci. In contrast, the PIP-box mutant does not co-localize with PCNA after damage exposure. Shown are representative micrographs, as well as quantifications of the percentage of PCNA foci co-localizing with HUWE1 and, reciprocally, the percentage of HUWE1 foci co-localizing with PCNA, in wild-type and PIP mutant-corrected cells. Only cells with more than five foci were analyzed. The average of three experiments, with standard errors, is shown. A total number of foci counted were as follows: for the HU experiment: 162 HUWE1-WT foci with 144 PCNA foci; 196 HUWE1-FF foci with 163 PCNA foci. For the UV experiment: 146 HUWE1-WT foci with 121 PCNA foci; 101 HUWE1-FF foci with 78 PCNA foci.

We next investigated if the PCNA-HUWE1 interaction is important for relieving replication stress. Wild-type HUWE1, but not the PIP-box mutant or the catalytically inactive mutant, could rescue the HU sensitivity of the HUWE1-knockout cell line (Fig 20A). Moreover,

the increased S-phase arrest, the reduced replication tract length, and the increased DNA breaks phenotypes could also be corrected by wild-type, but not by PIP-box mutant HUWE1 (Fig 20B-E). These results show that HUWE1 interaction with PCNA is essential for DNA damage tolerance and fork progression under replication stress.



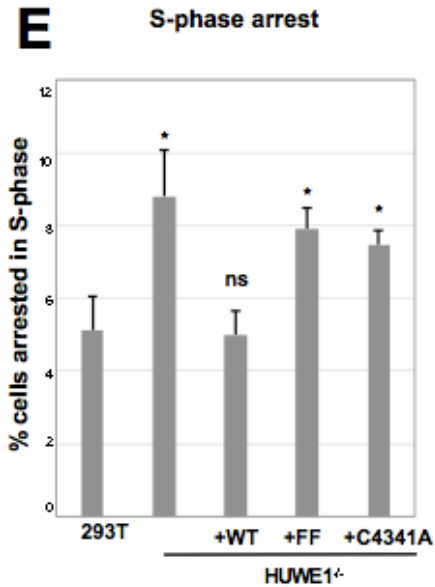


Figure 20. PCNA-HUWE1 interaction is required for relieving replication stress. A. Clonogenic assay showing that stable expression of wildtype HUWE1, but not of the PIP box mutant or the catalytic mutant, corrects the HU sensitivity of the HUWE1-knockout 293T cells. Shown is the average of 3 independent experiments \pm SD. If not indicated otherwise, the p-values shown specify the statistical significance relative to 293T (for the 0.5mM HU condition). P-values are: 0.0003 (293T vs HUWE1^{-/-}); 0.157 (293T vs HUWE1^{-/-} +WT); 0.0045 (293T vs HUWE1^{-/-} +FF); 0.0006 (293T vs HUWE1^{-/-} +C4341A); 0.047 (HUWE1^{-/-} +WT vs HUWE1^{-/-} +FF). B. The S-phase arrest phenotype is also rescued by wild-type, but not PIP-mutant or catalytic mutant HUWE1. S-phase arrested cells were quantified using the BrdU/PI bi-dimensional flow cytometry assay. Shown is the average of 3 independent experiments \pm SD. The p-values shown specify the statistical significance relative to 293T (0.018, 0.957, 0.015, and 0.024 respectively). A quantification of the same data, showing the percentage of cells arrested in S-phase, is shown (E). C. Normal replication tract length, quantified using the DNA fiber assay, is restored by expressing wild-type, but not PIP-mutant HUWE1, in the HUWE1-knockout 293T cells. The “n” numbers of fibers analyzed (pooled from three independent experiments), as well as the mean \pm SEM, are indicated on the graphs. If not indicated otherwise, the p-values shown specify the statistical significance relative to 293T. P-values are: 1.47×10^{-11} (293T vs HUWE^{-/-} samples), 0.094 (293T vs HUWE^{-/-} +WT samples), 1.40×10^{-7} (293T vs HUWE^{-/-} +FF samples), 1.22×10^{-7} (HUWE^{-/-} vs HUWE^{-/-} +WT samples), 0.679 (HUWE^{-/-} vs HUWE^{-/-} +FF samples). D. Alkaline Comet Assay showing that wild-type, but not the FF mutant can correct the breakage phenotype of HUWE1-knockout 293T cells. The “n” numbers of comet tails analyzed (pooled from three independent experiments), as well as the mean \pm SEM, are indicated on the graphs. The p-values indicated are: 4.18×10^{-43} (293T vs HUWE^{-/-} samples), 6.89×10^{-41} (HUWE1^{-/-} +WT vs FF samples), and 5.97×10^{-50} (HUWE1^{-/-} +WT vs C4341A samples). E. Rescue of the S-phase arrest phenotype by wild-type but not PIP mutant HUWE1. The same data as in Fig (B) are shown, but this time the percent of cells in S-phase is plotted. Shown is the average of three independent experiments \pm SD. The P-values shown (calculated using the t-test two-tailed, equal variance), indicate the statistical significance relative to 293T (0.015, 0.865, 0.011, and 0.016, respectively).

3.4. HUWE1 PROMOTES H2AX POST-TRANSLATIONAL MODIFICATIONS

We next examined if HUWE1 influences checkpoint signaling at stalled replication forks.

Surprisingly, we observed that the levels of monoubiquitinated histone variant H2AX are

significantly reduced in HUWE1-knockout HeLa and 8988T cells (Fig 21), suggesting that HUWE1 may act as a ubiquitin ligase towards H2AX. Indeed, *in vitro* ubiquitination reactions showed that HUWE1 can monoubiquitinate H2AX (Fig 22).

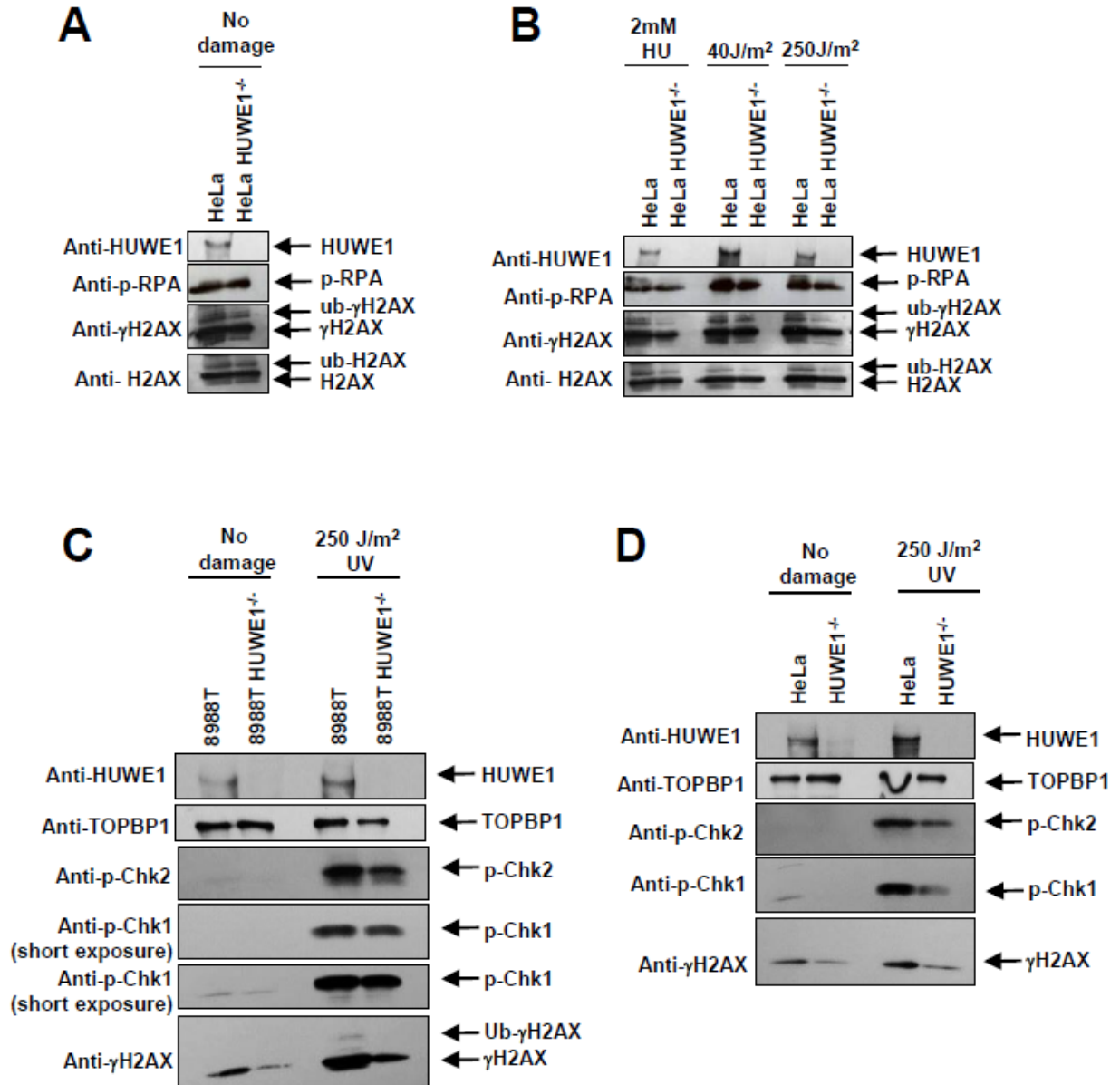


Figure 21. H2AX modifications are significantly reduced in HUWE1-knockout cells. A, B The impact of HUWE1 on H2AX phosphorylation and ubiquitination, under normal conditions (A) or upon induction of replication stress (2 mM HU for 18 h, or 2 h after exposure to 40 J/m² UV). Control or HUWE1-knockout HeLa cells were analyzed. C, D Western blots showing reduced γH2AX and phospho-Chk1 in HUWE1-knockout 8988T (C) and HeLa (D) cells.

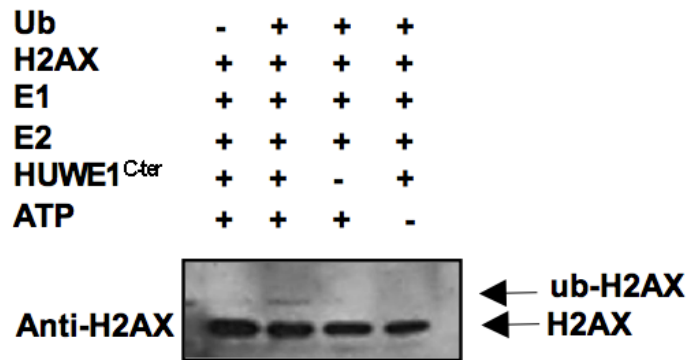


Figure 22. HUWE1^{C-ter} ubiquitinates H2AX in vitro. In vitro ubiquitination assay showing that recombinant HUWE1^{C-ter} can mono-ubiquitinate H2AX.

H2AX monoubiquitination was shown to promote its phosphorylation (Pan et al., 2011; Wu et al., 2011)—an important step in signaling at double strand breaks. In line with this, we observed that the level of phosphorylated H2AX (γ H2AX) is lower in HUWE1-knockout cells (Fig 21). Wild-type, but not PIP-box mutant HUWE1 could correct the H2AX mono-ubiquitination defect of HUWE1-knockout cells (Fig 23).

Moreover, HUWE1 knockout did not reduce γ H2AX ubiquitination in G1 cells treated with bleomycin—an agent that induces double strand breaks (Fig 24). These results indicate that HUWE1 monoubiquitinates H2AX at stalled replication forks.

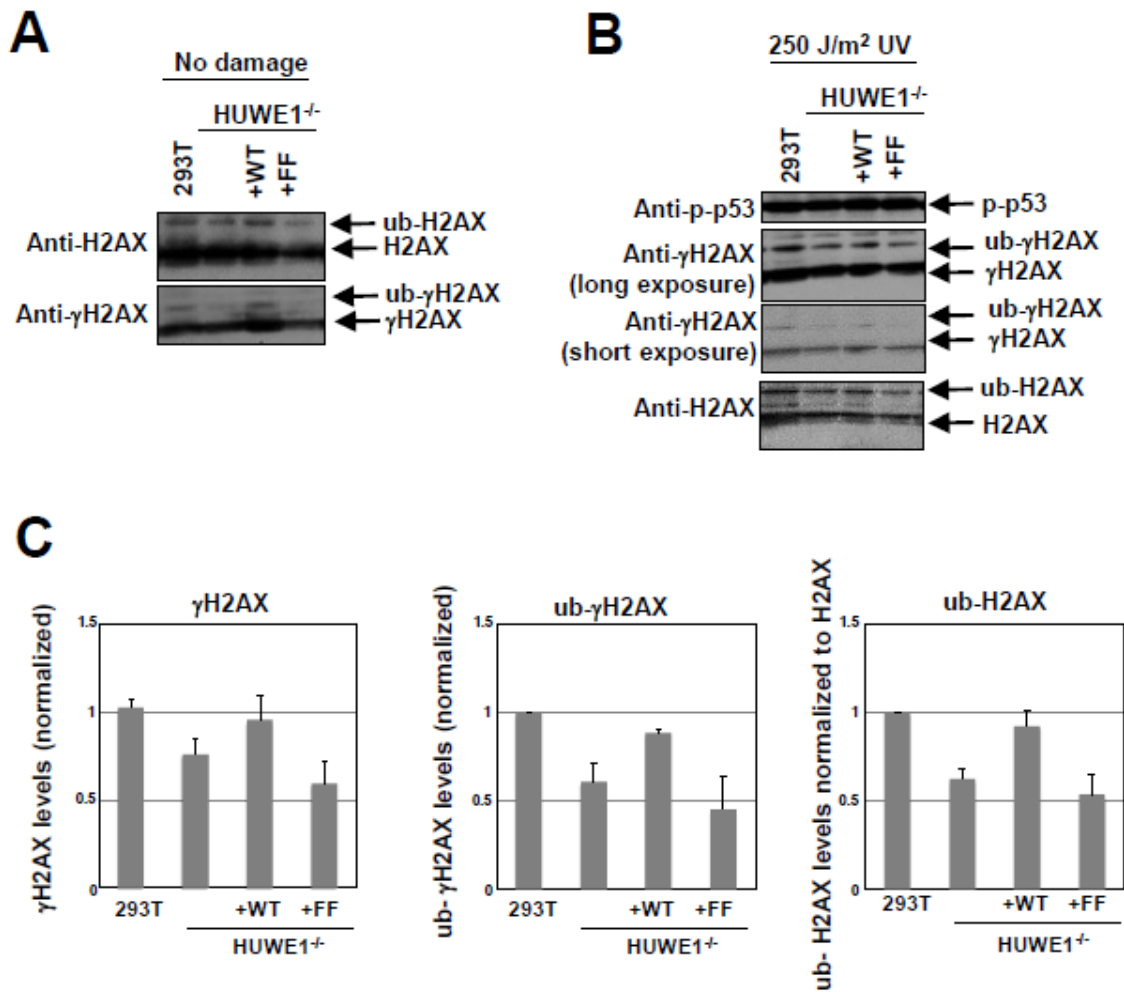


Figure 23. PIP-box mutant HUWE1 cannot rescue H2AX modification defects. A. Wild-type but not the PIP mutant can correct the defective H2AX ubiquitination in HUWE1-knockout 293T cells. B. Western blots showing reduced H2AX modification in HUWE1-knockout 293T cells, corrected by wild-type but not PIP mutant HUWE1. C. Quantification of γ H2AX (against input control), γ H2AX ubiquitination (against un-ubiquitinated γ H2AX), and H2AX ubiquitination (against unmodified H2AX). The average of three experiments is shown. Error bars represent standard deviations.

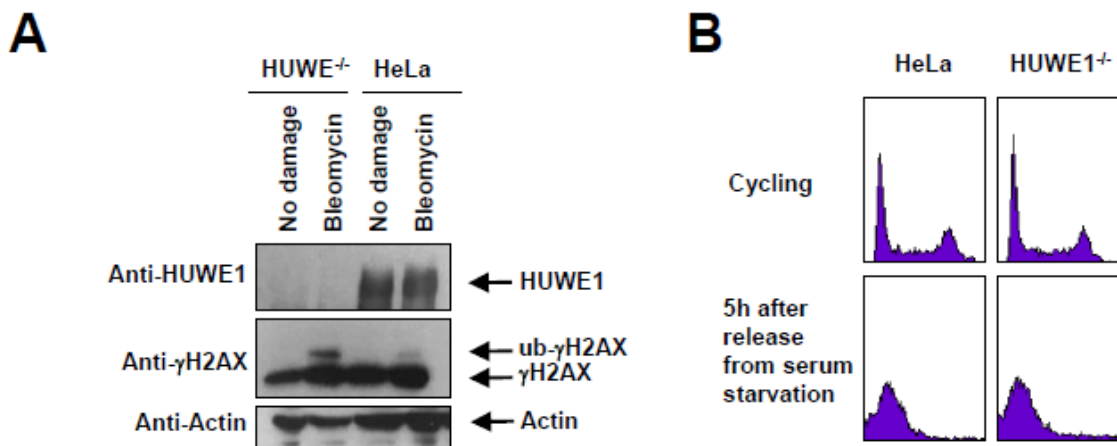


Figure 24. HUWE1-knockout does not reduce γ H2AX levels in G1. A. HUWE1 deletion does not reduce H2AX ubiquitination in G1. HeLa cells (wild-type or HUWE1-knockout) were arrested in G0 by serum starvation for 24 h, then released in normal media. Cells were analyzed 5 h later, when they reached G1. Cells were either treated with 5 μ M bleomycin for the last 2 h of the release or left untreated. B. Flow cytometry profile (PI staining) of cells at the harvest time point (5 h after release from serum starvation). The plot indicates that cells are uniformly in G1. For comparison, the normal profile of cycling cells is also presented.

To further confirm this, we investigated γ H2AX recruitment to common fragile sites. These difficult-to-replicate DNA regions act as endogenous replication stress elements, and are prone to fork collapse and breakage, which can be experimentally exacerbated by treating cells with the replication inhibitor Aphidicolin (Ozeri-Galai et al., 2012). As previously shown (Lu et al., 2013), γ H2AX can be detected by chromatin immunoprecipitation (ChIP) at the fragile site FRA3B in Aphidicolin-treated cells (Fig 25). This binding was dramatically reduced in HUWE1-deficient cells (Fig 25), indicating that HUWE1 is important for γ H2AX activity during the replication stress response.

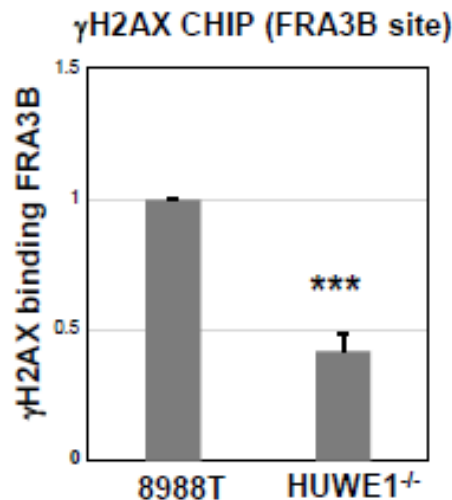


Figure 25. HUWE1-knockout reduces γ H2AX binding to CFS FRA3B. Chromatin immunoprecipitation showing that γ H2AX binding to the common fragile site FRA3B is reduced in HUWE1-knockout 8988T cells. Cells were treated with 600 nM aphidicolin for 24 h. Binding was quantified relative to input material. Shown is the average of four experiments \pm SD. P-value is 4.4×10^{-6} .

At double strand breaks, H2AX can be ubiquitinated by several ubiquitin ligases including RNF168 and RNF2/BMI1 (Mattiroli et al., 2012; Pan et al., 2011). Similar to HUWE1 depletion, knockdown of RNF168, RNF2, or BMI1 resulted in decreased γ H2AX ubiquitination (Fig 26),

and γ H2AX binding to FRA3B (Fig 27) following Aphidicolin treatment. Thus, efficient H2AX ubiquitination during the response to replication stress is achieved through the activity of multiple ubiquitin ligases.

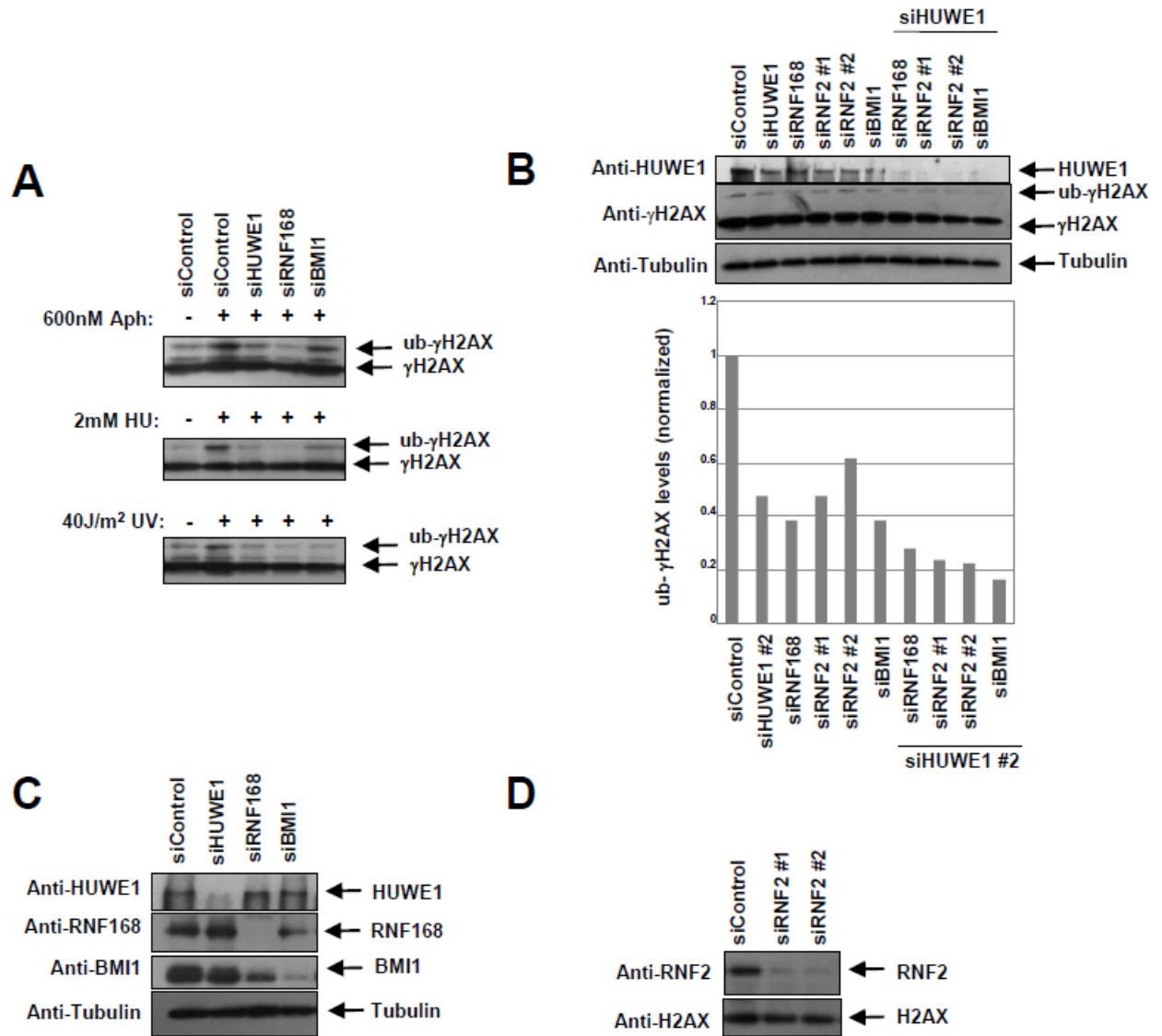


Figure 26. HUWE1 is a novel ubiquitin ligase for H2AX. A. Western blots showing that several H2AX ubiquitin ligases participate in γ H2AX ubiquitination following replication stress. HUWE1, RNF168, and BMI1 were knocked-down in HeLa cells. Cells were treated with 600 nM aphidicolin for 24 h, 2 mM HU for 24 h, or analyzed 2 h after exposure to 40 J/m² UV. B-D. Co-depletion of HUWE1 and other H2AX ubiquitin ligases enhances the H2AX ubiquitination defects. B. Representative Western blot experiment in HeLa cells. A quantification of this experiment is also shown. This result was replicated in three independent experiments. C, D. Western blots showing the efficient depletion of H2AX ubiquitin ligases.

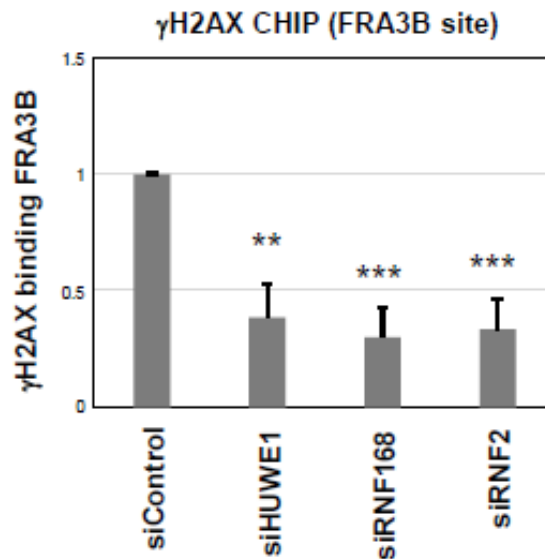


Figure 27. Knockdown of H2AX ubiquitin ligases reduce γ H2AX binding to FRA3B. HeLa cells were treated with 600 nM aphidicolin for 24 h. Binding was quantified relative to input material. Shown is the average of three experiments \pm SD. The P-values shown indicate the statistical significance relative to siControl (0.0016, 0.0005, and 0.0009, respectively).

We next wondered how H2AX ubiquitination by HUWE1 might promote replication fork stability. DNA fiber experiments indicated that knockdown of H2AX results in decreased replication tract length (Fig 28).

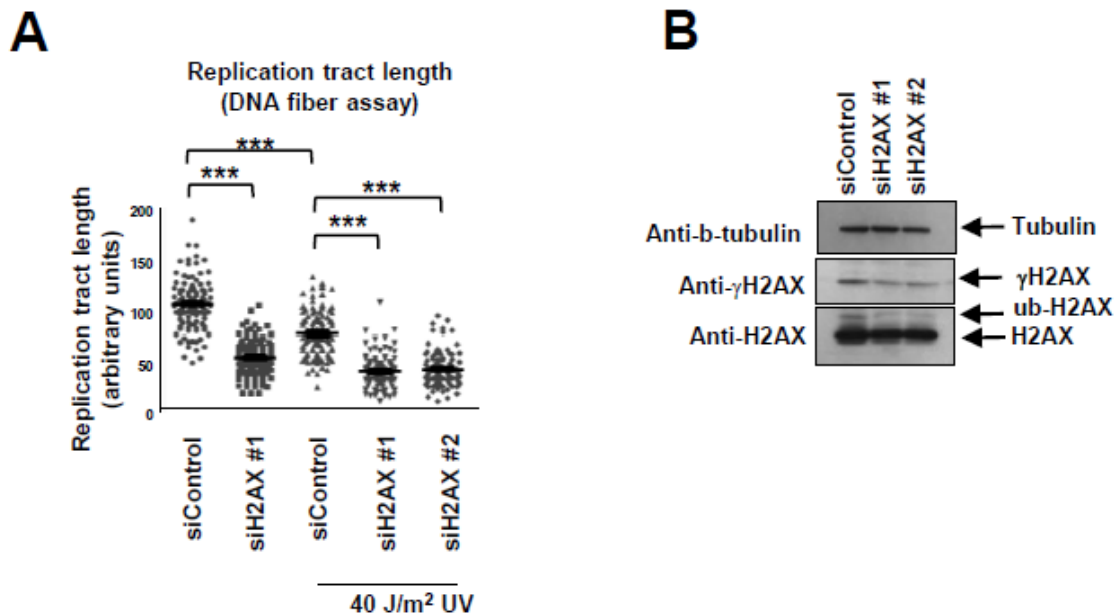


Figure 28. H2AX knockdown reduces replication tract length. A DNA fiber assay using HeLa cells with H2AX knockdown. Cells were either treated with 40 J/m² UV (analyzed 2 h later) or left untreated. The “n” numbers of samples are 101, 101, 106, 92, and 100, respectively (pooled from three independent experiments). P-values

(calculated using the t-test two-tailed, equal variance) are 1.19×10^{-40} (siControl vs. siH2AX, no damage samples); 5.6×10^{-15} (siControl, no damage vs. UV samples); 1.1×10^{-20} (siControl vs. siH2AX #1, UV samples); 9.01×10^{-32} (siControl vs. siH2AX #2, UV samples). B Western blot of HeLa cells showing the impact of H2AX siRNA oligonucleotides.

Indeed, it has been proposed that coating of damaged DNA by γ H2AX promotes the assembly of repair complexes (Hoeller and Dikic, 2009; Peterson and Côté, 2004). Among other repair factors, the recombination proteins BRCA1 and BRCA2 are known to promote restart of stalled replication forks (Pathania et al., 2014; Schlacher et al., 2011). Chromatin fractionation experiments showed reduced chromatin recruitment of BRCA1 and BRCA2 in HUWE1-knockout cells following induction of replication stress by HU (Fig 29). These results indicate that HUWE1-mediated ubiquitination of H2AX promotes recruitment of repair proteins to restart stalled replication forks.

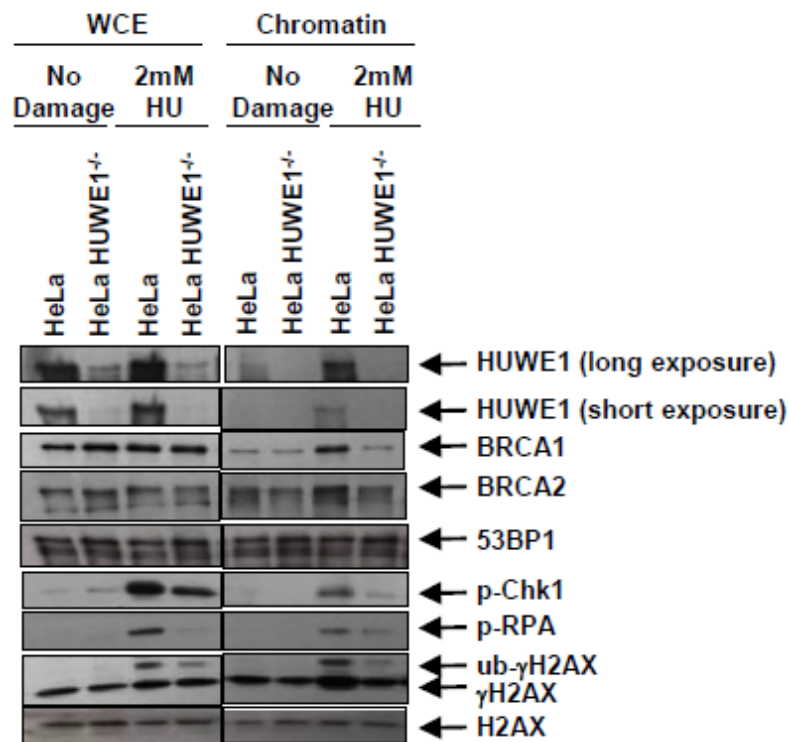


Figure 29. HUWE1 promotes the recruitment of repair proteins BRCA1 and BRCA2 to chromatin under replication stress. Chromatin fractionation experiments showing that HUWE1-knockout HeLa cells, treated with 2 mM HU for 24 h, fail to efficiently recruit BRCA1 and BRCA2 proteins to DNA. In contrast, 53BP1 chromatin association is not induced by HU treatment, and not affected by HUWE1 knockout, and thus can serve as loading control. Shown are blots of whole-cell extract (WCE) samples, representing input material, and of chromatin pellet samples.

Chapter 4. DISCUSSION

4.1. HUWE1 IS REQUIRED FOR GENOMIC INTEGRITY AND DNA DAMAGE TOLERANCE

HUWE1 is a promiscuous ubiquitin ligase that regulates many processes including cell cycle progression, DNA repair, and cell proliferation (Adhikary et al., 2005; Bernassola et al., 2008; Chen et al., 2006; Chen et al., 2005; de Groot et al., 2014; Hao et al., 2012; Inoue et al., 2013; Jang et al., 2014; Kurokawa et al., 2013; Liu et al., 2012; Maltseva et al., 2013; Pervin et al., 2011; Peter et al., 2014; Qi et al., 2013; Schaub and Cleveland, 2014; Vaughan et al., 2015; Wang et al., 2014). We created, for the first time, HUWE1-knockout human cells using the CRISPR/Cas9 technology. Human embryonic kidney 293T cells with HUWE1 knockout showed a dysregulation of cancer-related pathways through RNA-seq analyses (Fig 7), indicating an important role for HUWE1 in cellular transformation. This prompted us to investigate if HUWE1 directly impacts genomic integrity, a well-described enabling characteristic of cancer cells.

HUWE1 has been reported to affect a number of genome stability mechanisms. HUWE1 degrades the pre-replication complex member Cdc6 after exposure to DNA damaging agents, possibly to suppress firing of late or dormant replication origins (Hall et al., 2007; Hall et al., 2008). HUWE1 also controls base excision repair by initiating the degradation of DNA polymerase Pol β , and its knockdown was reported to result in slightly increased rates of repair of hydrogen peroxide-induced DNA damage (Markkanen et al., 2012; Parsons et al., 2009). Finally, HUWE1 was shown to degrade BRCA1, and HUWE1 knockdown in breast cancer MCF10F cells resulted in increased resistance to double strand break-inducing agents such as ionizing radiation (Wang et al., 2014). In contrast, our results clearly indicate that HUWE1 knockdown or knockout confers sensitivity to replication fork stalling agents HU and UV (Fig 6, 8-10). Moreover, our DNA fiber

experiments showed a reduction in progression of replication forks both with, and without exogenous DNA damage treatment (Fig 8J and K, 9I, 5E and F). Finally, analysis of cell cycle progression by co-staining for BrdU incorporation and DNA content (Fig 8D and I, 9D-H) identified a specific increase in the population of cells arrested in S-phase. This profile is consistent with increased replication stress in HUWE1-depleted cells. These results argue that HUWE1 plays an important role in the response to replication stalling, which is separate from the previously described activities in degrading DNA repair proteins. The reduced fiber tract length in HUWE1-deficient cells, would suggest that these cells spend a longer time in S-phase. However, the number of cells with S-phase DNA content is similar (but a larger proportion of those are BrdU-negative). These findings may suggest an additional function of HUWE1 in regulating entry into S-phase.

4.2. HUWE1 IS A NOVEL PCNA-BINDING PARTNER

PCNA is a homotrimeric ring-shaped sliding clamp that plays a critical role in DNA replication and repair (Moldovan et al., 2007). Here, we report for the first time that HUWE1 associates with PCNA via the PIP box **QPAVEAFF**, and is associated with replication forks. The co-localization of PCNA with HUWE1 after DNA damage treatment may indicate that HUWE1 is specifically recruited to stalled forks, but it may also simply mark the sites of repair DNA synthesis. PIP-box mutations abolished this localization and could not correct the replication stress phenotypes of HUWE1-knockout cells, indicating that PCNA interaction is essential for this novel activity of HUWE1. HUWE1-knockout cells also have a growth defect (not shown), again illustrating the broad, pleiotropic impact of HUWE1. While we could correct the HU sensitivity phenotype by expression of exogenous Myc-HUWE1 (Fig 18-20), the growth defect was, in fact, not corrected—potentially indicating a role for transcriptional regulation of HUWE1 expression.

At stalled replication forks, PCNA monoubiquitination results in the recruitment of specialized low fidelity TLS polymerases (Bienko et al., 2005; Guo et al., 2009). Because of our findings that the E3 ligase HUWE1 interacts with PCNA, and that HUWE1 knockdown results in sensitivity to replication fork stalling agents HU and UV, we initially speculated that HUWE1 might monoubiquitinate PCNA to promote DNA damage tolerance and lesion bypass. However, despite our intense efforts, we did not detect a reproducible reduction in PCNA monoubiquitination in HUWE1-knockout cells.

4.3. HUWE1 PROMOTES H2AX MODIFICATIONS AT STALLED FORKS

The role of H2AX at sites of double strand breaks has been extensively studied (Ciccia and Elledge, 2010). H2AX phosphorylation by ATM initiates the damage signaling process by recruiting MDC1, one of the earliest proteins in the signaling cascade. H2AX is also ubiquitinated, which promotes recruitment of repair proteins. Blocking H2AX monoubiquitination also results in reduced γ H2AX signal at double strand breaks, but how H2AX ubiquitination promotes its phosphorylation is still unclear (Pan et al., 2011; Wu et al., 2011). It was recently shown that γ H2AX also accumulates at stalled replication forks, in a process that depends on the ATR kinase, the counterpart of ATM for signaling replication stress (Ewald et al., 2007; Gagou et al., 2010; Sirbu et al., 2011; Ward and Chen, 2001). The exact role of γ H2AX at stalled forks is much less understood compared to its activity at double strand break sites. Interestingly, MDC1 was shown recently to be recruited to stalled forks, where it interacts with TopBP1, a known ATR activator, suggesting that γ H2AX may be important for amplification of the checkpoint signal at stalled forks (Wang et al., 2011).

Surprisingly, HUWE1-knockout cells had reduced γ H2AX levels (Fig 21) even though they showed increased replication stress (Fig 6, 8-10). This suggests that H2AX phosphorylation

at stalled replication forks is impaired in the absence of HUWE1. HUWE1 was previously shown to ubiquitinate H2A during spermatogenesis (Liu et al., 2007; Liu et al., 2005). We noticed that HUWE1-knockout cells have reduced H2AX mono-ubiquitination (Fig 21). Importantly, recombinant HUWE1 could monoubiquitinate H2AX *in vitro* (Fig 22). These results indicate that HUWE1 is a novel ubiquitin ligase for H2AX. The reduced γ H2AX levels in HUWE1-knockout cells may thus be due to the ubiquitination defect, echoing the H2AX ubiquitination-phosphorylation link previously described at double strand breaks. Since we could correct the H2AX ubiquitination defect using wild-type, but not PIP-mutant HUWE1 (Fig 23), and we observed no defect in H2AX ubiquitination in HUWE1-knockout cells during G1 (Fig 24), we hypothesize that HUWE1 is responsible for H2AX ubiquitination at stalled forks. However, it is possible that HUWE1 also acts on H2AX under other conditions as well. Interestingly, a recent publication reported a role for HUWE1 in regulating levels of H2AX through multiubiquitination-mediated degradation under steady-state *versus* ionizing radiation exposure (Atsumi et al., 2015). We have not obtained any evidence for multi-ubiquitination in our *in vitro* ubiquitination assay, and did not observe differences in H2AX levels in our HUWE1-knockout cells under the replication stress conditions that our studies focused on. Further complicating this picture, we found that multiple E3 ligases are involved in replication stress-induced H2AX ubiquitination (Fig 26 and 27). In line with this, we noticed that, in 293T cells, the γ H2AX deficiency is a transient phenotype: after several passages, HUWE1 knockout cells tend to recover normal γ H2AX levels (not shown). It is likely that back-up pathways become activated to restore DNA damage signaling. Future studies aiming at understanding the complex interplay between the activities regulating the activity of H2AX in the response to DNA damage and replication stress are thus very important.

Replication stress is a major threat to genomic integrity, as evidenced by the hypersusceptibility to breakage of common fragile sites. Aphidicolin-induced γ H2AX binding to

fragile sites is severely reduced by HUWE1 depletion (Fig 25 and 27), showing that HUWE1 is directly involved in protecting replication forks. This is further highlighted by our findings that HUWE1 promotes the recruitment of repair proteins such as BRCA1 and BRCA2 to chromatin under replication stress conditions (Fig 29). Homologous recombination and other DNA repair mechanisms have been shown to participate in restarting stalled replication forks (Gaillard et al., 2015; McGlynn and Lloyd, 2002; Petermann et al., 2010; Yeeles et al., 2013). In particular, the involvement of BRCA1 and BRCA2 in promoting replication fork stability has been well documented (Pathania et al., 2014; Schlacher et al., 2011). Our results thus indicate a model wherein, following replication fork stalling, HUWE1 promotes ubiquitination and phosphorylation of H2AX, which in turn recruits repair complexes to repair and/or restart the damaged fork (Fig 30).

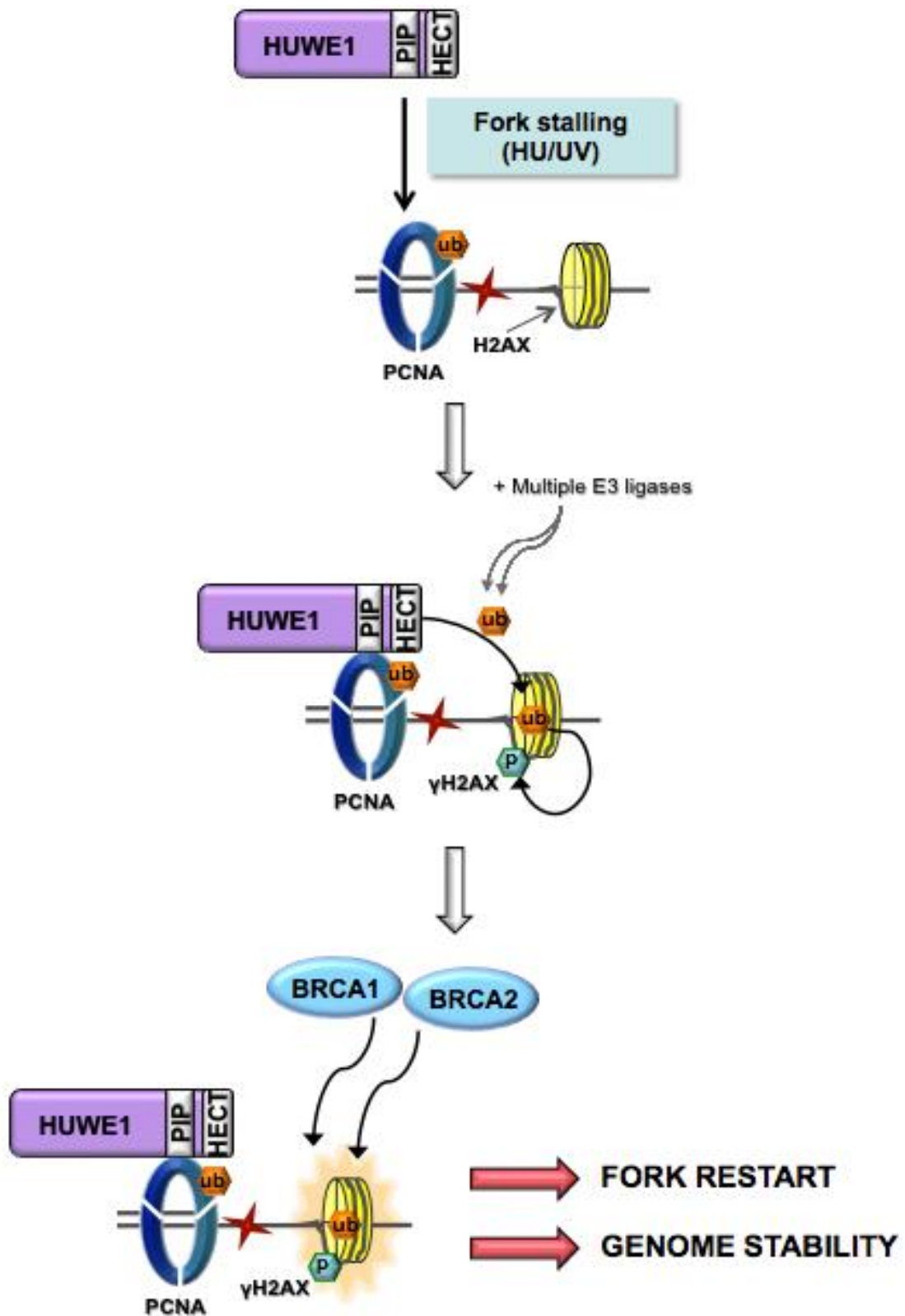


Figure 30. HUWE1 comes to the rescue at stalled forks. Under replication stress, HUWE1 is recruited to stalled forks through PCNA, mediated by its PIP-box motif. Once bound to PCNA, HUWE1 monoubiquitinates H2AX to promote its phosphorylation and γ H2AX signaling to recruit repair complexes, including BRCA1 and BRCA2 to repair and/or restart damaged forks. Red star represents replication obstacle.

4.4. CONCLUDING REMARKS

4.4.1. *New Directions for HUWE1*

Faithful propagation of the genome to progeny is fundamental for preserving genome integrity. The genome is under persistent attack by endogenous and exogenous insults during replication. To deal with these attacks, the cell evolved mechanisms to bypass these obstacles and complete replication. We describe a mechanism in which HUWE1 is a regulator of replication stress. HUWE1 is recruited to stalled forks through its PIP-box interaction with PCNA and monoubiquitinates H2AX to initiate the DNA damage response and promote the recruitment of repaired proteins to restart stalled forks.

Our work opens up new interesting research directions for HUWE1. We demonstrate that the proper localization of HUWE1 depends on PCNA; however, the regulation behind the recruitment of HUWE1 to replication forks remains unexplored. The recruitment of many, if not all, proteins to replication forks involved in the DNA damage response is regulated by posttranslational modifications and/or accessory proteins (O'Connor et al., 2013). This could also be the case for HUWE1. One could test this by detecting expression levels of endogenous or exogenous posttranslational modifications by Western blot, with or without a damage-specific context. If any forms of modifications are detected, the targeted residues can be identified through mass spectrometry, and mutated to test whether these modifications affect HUWE1 recruitment to chromatin. These studies can then be expanded to different stimuli or under different DNA damage response pathways.

iPOND coupled with mass spectrometry is a powerful tool to detect which complexes form at the replication fork. To test whether HUWE1 is recruited with accessory proteins, one could use this technique to identify what complexes HUWE1 is involved in at the replication fork under

stressed and unstressed conditions, and whether other proteins within this complex chaperone HUWE1 to these sites.

BRCA1 and BRCA2 have functions at stalled forks that are independent of their well-described roles at double strand breaks. We find that HUWE1 is required for BRCA1 and BRCA2 recruitment to stalled forks under HU treatment, but the functions of the recruited BRCA1 and BRCA2 proteins are unknown. BRCA1 and BRCA2 have been shown to stabilize stalled forks by preventing MRE11-mediated nascent DNA degradation, and BRCA2 has been shown to stabilize RAD51 filaments on ssDNA (Schlacher et al., 2011; Ying et al., 2012). BRCA1 participates in a recombination-based fork restart mechanism at stalled forks but, interestingly, this mechanism is distinct from HDR that occurs at DSB, suggesting a divergent role of BRCA1 in HDR at stalled forks versus DSB (Willis et al., 2014). BRCA1 actually possesses an intrinsic binding affinity for ds/ssDNA junctions which are structures that are found at stalled forks (Paull et al., 2001). Under UV exposure at stalled forks, BRCA1 has been shown to be involved in post-replicative gap filling through a NER-independent mechanism in multiple ways: by activating the G2/M checkpoint, directing the RFC complex formation at stalled forks, and controlling the recruitment of pol η to damaged sites. Indeed, BRCA1 plays a critical role in replication stress as its haploinsufficiency has been shown to have a profound effect on its replication stress functions, while having no effect on its roles in HDR at DSBs (Pathania et al., 2014; Pathania et al., 2011). Due to the multiple functions of BRCA1 and BRCA2 at stalled forks, it is an interesting direction to pursue what functional outcomes of BRCA1/2 are promoted by HUWE1 at stalled forks, whether it is fork stabilization, fork restart, HDR, and/or PRR.

The DNA fiber assay is a unique tool in that, in addition to measuring elongating forks, it also allows the user to measure stalled fork stabilization and fork restart. One could use the DNA fiber assay to test whether BRCA1 and BRCA2 recruited by HUWE1 promote stabilization of

stalled forks by pulse-labeling cells with a nucleoside analog, and then stalling forks for an extended period of time with HU. Fork destabilization will result in degradation, which can be visualized by a shortened replication tract length compared to stabilized forks. Moreover, Mre11 recruitment to chromatin can also be tested through iPOND and chromatin fraction studies, to examine whether HUWE1 affects its binding and degradation of nascent DNA. To test fork restart, one can use the same DNA fiber set up as mentioned but, after prolonged HU treatment, remove the agent and incubate cells with a second thymidine analog to measure the ability of the cell to restart stalled forks. Using these techniques would provide insight to how HUWE1-mediated recruitment of these proteins protects stalled forks. To test whether active repair is occurring through HUWE1, one could again use the versatile iPOND coupled with mass-spectrometry to examine what other proteins are recruited to stalled forks through HUWE1 in addition to BRCA1 and BRCA2, and tease out what active mechanisms are occurring at the fork.

Furthermore, our iPOND studies show that HUWE1 associates with the replisome even during unperturbed S-phase. This could be due to HUWE1 facilitating DNA replication in the presence of intrinsic cellular sources of replication stress, but could also suggest that HUWE1 might be a functional component of the replisome to assist in facilitating normal DNA replication. To test whether HUWE1 is a functional part of the replisome, one could use the iPOND technique with a thymidine chase to test whether HUWE1 moves along with the replication machinery or whether HUWE1 is recruited to maturing chromatin to address DNA lesions.

HUWE1 has been reported to affect other DNA repair pathways such as BER and HR through its substrates. These repair pathways involve DNA synthesis, facilitated by PCNA. Because we see that HUWE1 interacts with PCNA and, because PCNA is used in other replication-dependent repair processes such as nucleotide excision repair (NER) and mismatch

repair (MMR), it is reasonable to speculate that HUWE1 might play a role in these pathways as well. To test this, cells can be treated agents that induce the formation of bulky DNA adducts (which are resolved through NER) or damaged bases (which are resolved by MMR) and test whether HUWE1 is involved in the repair of these damages. Under these conditions, HUWE1-dependent phenotypes can be examined, the requirement for HUWE1-PCNA interaction in these contexts can be explored, and future studies can be aimed at teasing out which step of these repair processes include the participation of HUWE1.

4.4.2. PCNA and HUWE1 as Therapeutic Targets

4.4.2.1. PCNA and Human Disease

Reflecting its role in promoting genomic stability, several close companions of PCNA have been found to be associated with cancer predisposition syndromes, including ATAD5 for ovarian cancer (Kuchenbaecker et al., 2015), and SPARTAN for hepatocellular carcinoma (Lessel et al., 2014). Moreover, PARI expression can predict the tumor response to therapy, presumably through its HR-inhibiting activity (Pitroda et al., 2014). Mice expressing the K164R mutant as the only source of PCNA show altered somatic hypermutation at the IgG loci (Roa et al., 2008), suggesting that human B-cell deficiency syndromes may also be caused by defective PCNA ubiquitination. However, until very recently, no PCNA mutation has been reported in human disease, reflecting perhaps the fact that most mutations are expected to destroy the complex fold of PCNA and thus result in inactivation of this essential protein.

A recent report presented the first human syndrome caused by PCNA mutation (Baple et al., 2014). The patients, from a single extended family, share a homozygous missense PCNA mutation, Ser228Ile. Ser228 lies close to the IDCL and the mutation results in a large conformational change that affects interactions with PIP-box proteins including Fen1, DNA ligase

1, and XPG (Duffy et al., 2016). For reasons still not completely clear, the mutation affects DNA repair rather than DNA replication functions of PCNA (Green et al., 2014). This helps explain why the patients with this mutation survive, but suffer from immunodeficiency, photosensitivity, and neurodegeneration symptoms (including: hearing loss, ataxia, cognitive decline, and brain defects). Indeed, DNA repair-related syndromes, such as ataxia telangiectasia (ATM mutations), exhibit clinical characteristics that include neurodegeneration (Rass et al., 2007). More recently, homozygous missense mutations in the PCNA binding partners TRAIP and PARP10 have been associated with microcephaly (Harley et al., 2016; Shahrour et al., 2016). Similar to the PCNA mutations, cells from these patients show a strong DNA repair defect but a relatively minor proliferation defect.

Inherited DNA repair defects result in increased mutation rates, giving rise to cancer predisposition syndromes. How DNA repair defects cause neurodegeneration is less clear. During early neurogenesis, the rapid proliferation of nervous tissue may require efficient fork restart mechanisms such as TLS to deal with replication stress. At later stages, once brain cells become quiescent they are uniquely challenged by the high levels of radical oxygen species generated by their increased metabolic rates. Coupled with the high transcriptional demand and their non-proliferative status which does not allow tissue regeneration, this makes brain cells uniquely susceptible to DNA repair defects, and explains how inability to repair DNA damage results in loss of neurophysiological functions (Rass et al., 2007).

4.4.2.2. Targeting PCNA in Cancer Therapy

Because of its essential role in promoting proliferation, PCNA is an obvious target for cancer therapy (Wang, 2014). However, since PCNA is not an enzyme and instead acts through its protein interactions, it has been very difficult to design drugs that inhibit PCNA. In recent years,

a number of studies identified small molecules that block PCNA-Pol δ interaction or PCNA trimer formation, with proliferation inhibitory effects *in vitro* (Punchihewa et al., 2012; Tan et al., 2012; Zhao et al., 2011) (Table 3). Moreover, a cell permeable peptide containing the APIM motif, which blocks binding of partners with the APIM motif, showed promising results in treating myeloma and bladder cancer, in mouse xenograft studies (Müller et al., 2013). These studies suggest that PCNA targeting in cancer may not be far away (Wang, 2014).

<i>Inhibitor</i>	<i>Properties</i>	<i>Mechanism of action</i>	<i>Effects</i>
T2AA (Punchihewa et al 2012)	T3 thyroid hormone derivative	- Binds the PCNA cavity that interacts with PIP-box motifs; - Interferes with PCNA-PIP-box interactions; - Does not affect PCNA-DNA binding.	Inhibits PCNA interaction with p21 (<i>in vitro</i>) and pol δ (in cells). Treatment results in inhibition of cell growth and proliferation, increased replication stress with activation of p-Chk1 and γ H2AX, and reduced TLS. Combination with cisplatin increases cellular DNA damage and reduces cell growth in U2OS cells.
PCNA-I1 (Tan et al 2012)	Small molecule inhibitor	- Stabilizes the PCNA trimer by binding Arg146 through an O-N hydrogen bond on one monomer of PCNA, and to Asp86 through an N-O hydrogen bond on its adjacent monomer.	Treatment reduces PCNA binding to chromatin, possibly through trimer stabilization. Inhibits growth in various human and mouse cancer cell lines with a potency ~9-fold higher than in untransformed cells. Leads to G1 accumulation in the first 24 hours and an S and G ₂ /M arrest by 72 hours in PC-3 cells.
ATX-101 (Müller et al 2013)	APIM-containing peptide	Interferes with PCNA-APIM motif interactions.	Induces apoptosis in multiple myeloma and primary cancer cells in a caspase-dependent but cell cycle-independent manner. Potentiates the cytotoxicity of Melphalan in multiple myeloma cell lines and in a xenograft multiple myeloma mouse model.
R9-caPeptide	Cell permeable peptide	Interferes with PCNA-PIP motif interactions.	Preferentially targets PCNA-protein interactions in cancer cells over normal cells. Disrupts PCNA association to chromatin and pol δ ,

(Smith et al 2015 and 2016)			inhibiting DNA replication <i>in vitro</i> and cell growth. Enhances sensitivity to cisplatin in cancer cells. Inhibits tumor growth in mice.
Y211F Peptide (Zhao et al 2011)	Peptide covering the Y211 region of PCNA	Inhibits PCNA phosphorylation at Y211.	Induces S-phase arrest, inhibition of DNA synthesis, and enhanced cell death in human prostate cancer cell lines. Decreases tumor growth in PC3-derived xenograft tumors in nude mice

Table 4. PCNA inhibitors that are being tested for cancer therapy.

4.4.2.3. HUWE1 in Genome Stability and as a Therapeutic Target

The functions of HUWE1 in tumorigenesis remain controversial. HUWE1 is overexpressed in various cancer cell lines and its dysregulation has been found in different cancer types (Adhikary et al., 2005; Chen et al., 2005; Confalonieri et al., 2009; Liu et al., 2012; Yoon et al., 2005; Zhao et al., 2008). This evidence suggests that HUWE1 promotes tumorigenesis, which is further supported by studies demonstrating that HUWE1 promotes oncoprotein activation and cellular proliferation through c-Myc (Adhikary et al., 2005), as well as its through its role in promoting the degradation of tumor suppressor p53 (Chen et al., 2005) and DNA repair proteins. Contradictingly, HUWE1 also demonstrates tumor-suppressive properties, reflected by its activity on anti-apoptotic protein Mcl-1 (Zhong et al., 2005) and its role in suppressing Ras-driven tumorigenesis in a mouse model by preventing c-Myc/Miz1-mediated downregulation of p21 and p15 (Inoue et al., 2013).

Our studies clearly demonstrate that HUWE1 plays a critical role in preventing genome instability and tumorigenesis by resolving replication stress. Not only did we employ siRNA technology to target HUWE1 for our phenotype studies as other groups have done, but we were the first to genetically silence HUWE1 through the CRISPR/Cas9 system. Using both systems, we were able to observe consistent phenotypes of HUWE1 in protecting genome stability. The

CRISPR/Cas9 system provides advantages over siRNA-mediated knockdown of proteins; it completely abolishes protein expression and has less off-target effects, a major limitation of using siRNA-targeted downregulation. This tool allows for cleaner phenotypic studies. Our findings on HUWE1 contradict some published reports in the literature. We believe that these differences may be due to off-targeting effects by siRNAs used in these studies, differences in cell line specificity, as well as different types of stimuli and damages used in these reports.

Although HUWE1 plays a pivotal role in preventing tumorigenesis in normal cells by relieving replication stress, a transformed cell can hijack this process for its survival. Many chemotherapeutic agents take advantage of challenging replication of tumor cells by creating replication barriers and inducing DNA breaks and apoptosis. Cisplatin, a commonly used chemotherapeutic agent that induces crosslinks, is a prime example. A caveat of using these types of agents is that the cancer cell is able to acquire resistance by activating mechanisms that enable bypass of these obstacles or by activating a repair mechanism to fix damaged or broken forks. It is likely that HUWE1 can facilitate bypass of replication obstacles in transformed cells. Thus, in this context, HUWE1 can play an oncogenic role and promote the survival and division of cancer cells. This could explain the oncogenic phenotypes of HUWE1 observed in the literature, and why HUWE1 is dysregulated in various cancer types.

With this perspective, HUWE1 would be a desirable target for cancer therapy. Indeed, newly highly specific HUWE1 small molecule inhibitors have been developed to examine whether HUWE1 can be exploited therapeutically (Peter et al., 2014). Studies using these small molecular inhibitors in colorectal cancer have demonstrated that HUWE1 promotes the growth of colorectal cancer cell lines in culture and in xenograft mouse models. Application of these small molecular inhibitors of HUWE1 resulted in inhibition of colorectal cancerous tissue culture and tumor growth size, and downregulation of MYC target gene activation. The inhibition of

MYC-dependent transactivation was only observed in the colorectal cancer cells, and spared in stem and normal colon epithelial cells, suggesting that HUWE1 can be an effective “druggable target” for chemotherapy. While these inhibitors demonstrate a global effect in HUWE1-dependent MYC gene-transactivation, it would be useful to create small molecule inhibitors that selectively target the PIP-box and interrupt HUWE1-PCNA interaction to compromise the ability of a cancer cell to tolerate sources of replication stress. Development of such inhibitors could be used alone or in combination with other replication stress-inducing chemotherapeutic agents to fight cancer.

Since its discovery, HUWE1 is continuing to emerge as a critical protein involved in a plethora of cellular processes that affect cell proliferation, cell survival, neuronal development, DNA repair, and genomic stability. HUWE1 is a necessary protein for the development and viability of an organism, and mutations of HUWE1 or aberrant protein levels result in profound pleiotropic phenotypes as well as death. Although the understanding of the important functional mechanisms of HUWE1 and the library of its known substrates are expanding, many unknowns remain. The significant impact of HUWE1 in cellular integrity demonstrates the necessity for continued studies on this pivotal protein. Understanding its underlying cellular mechanisms will provide insight in the regulation of many cellular processes that HUWE1 is involved in, and will allow us to exploit this important protein therapeutically.

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