Doctoral Thesis

Maintenance of chromatin integrity by novel Cullin 4-based ubiquitin ligases

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Maintenance of chromatin integrity by novel Cullin 4-based ubiquitin ligases

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presented by
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2015
For my Mother and Father.
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Summary

Cullin 4 (CUL4) is a scaffold protein capable of forming a multitude of E3 ubiquitin ligases. It is an important regulator of DNA replication and the DNA damage response. However, the molecular mechanisms of only a handful of Cullin 4-RING E3 ubiquitin ligases (CRL4s) are well understood. In an effort to fill this gap in knowledge, an RNAi-based live-cell imaging screen was carried out in the Peter group to identify new CUL4 adaptors that function in S phase. This led to the identification of two CUL4 adaptors WDR23 and WDR42a which are required for S phase progression and the characterisation of their functions became the focus of this PhD work.

DNA replication requires a steady supply of nascent histones for chromatin assembly. Changes in histone dosage have detrimental effects on the cell cycle, genome stability and development. Here, we present functional characterisation of CRL4\textsuperscript{WDR23} showing that the ubiquitin ligase performs a novel function in stimulation of histone mRNA processing.

The stem-loop binding protein (SLBP) is an important regulator of histone mRNA processing and translation, and this protein was found to be a ubiquitylation target of CRL4\textsuperscript{WDR23}. When the ubiquitylation activity of CRL4\textsuperscript{WDR23} is disengaged through disruption of its complex formation or when WDR23 is downregulated in cells, unprocessed histone mRNAs were
Summary

strongly accumulated and the pool of histone proteins was depleted. The effect of this on cells was a significant reduction in replication fork speed, S phase delay through CHK1 activation and grossly impaired cell growth.

After replication begins, DNA double strand breaks (DSBs) that appear can be accurately repaired by homologous recombination using available nascent DNA as the template. The phenotypic characterisation of WDR42a depletion showed that its function is required for efficient homologous recombination repair of DSBs. Upon WDR42a depletion, this repair activity is reduced leading to strong accumulation of persistent DNA DSBs that activate the DNA damage checkpoint and trigger apoptosis.

The newly identified functions of the two CUL4 adaptors, WDR23 and WDR42a, in histone mRNA processing and homologous recombination repair respectively represent new insights into the range of regulatory roles performed by CUL4-based E3 ligases. These findings further highlight the importance of CUL4 in DNA replication and also emphasise the functional diversity of CRL4s in maintaining a normal S phase. Using the range of substrate specific adaptors at its disposal, CUL4 is able to juxtapose and integrate different functionalities to fulfil its overall goal as a guardian of chromatin integrity. Ultimately, the characterisation of more CUL4 adaptors, including the two presented here, will lead us to a better understanding of how CUL4 maintains normal replication and genome stability.
1 Introduction

1.1 Ubiquitylation and Cullin 4 RING E3 ligases

1.1.1 The ubiquitylation cascade

Ubiquitin is a small, highly conserved protein of 76 amino acids found in eukaryotic cells. It acts as a molecular switch and is attached specifically and reversibly to substrate proteins by the ubiquitin cascade and targets the substrate for regulation or degradation (Figure 1.1). This pathway involves the consecutive activities of three enzymes, the E1 activating enzyme, E2 conjugating enzyme and finally the E3 ligase which transfers ubiquitin onto lysine residues on substrates.

There are various different forms of ubiquitylation which occur in cells and they signal for different outcomes for substrates. Ubiquitin can be attached as single moieties in mono or diubiquitylation modifications on substrates. As there are seven lysines (K6, K11, K29, K33, K48 and K63) found in ubiquitin itself, any of these residues can be utilised for additional conjugation and propagation of ubiquitin chains. As a result chains of different linkages are created. As an example of the regulatory multifunctionality of ubiquitylation, lysine 48 (K48)-linked polyubiquitin chains label proteins for degradation by the 26S proteasome, while
monoubiquitylation and lysine 63 (K63)-linked polyubiquitin chains dictate other regulatory functions. A compelling example of the non-proteolytic function of ubiquitylation is provided by monoubiquitylation and K63 poly-ubiquitylation of PCNA on the same residue to switch between error-prone versus error-free replication block bypass (Hoege et al., 2002).

The attachment and removal of ubiquitin are catalysed by proteins which contain ubiquitin-binding domains (UBDs) and are termed ubiquitin-binding proteins (UBPs). Another class of UBPs recognise different ubiquitylations on substrates and function to decipher these molecular signals, as found in Figure 1.1 (Hurley et al., 2006).

![The ubiquitin conjugation pathway.](image)

**Figure 1.1 | The ubiquitin conjugation pathway.**

The ubiquitin conjugation pathway consists of three enzymes, the E1 activating enzyme, the E2 conjugating enzyme and the E3 ligase which transfers the ubiquitin onto the substrates. There are various forms of ubiquitylation events which differ in number and linkage of ubiquitin moieties. These are recognised by specific UBPs which relay the molecular signals.
1.1.2 Cullin-based E3 ubiquitin ligases

In higher eukaryotes, there are more than 1,000 E3 ligases belonging to two major families, the HEC (homologous to E6-AP C-terminus)-domain family and the RING (really interesting new gene) family. In RING E3 ubiquitin ligases, the RING domain functions to bring the ubiquitin-charged E2 enzyme into contact with the substrate to stimulate ubiquitin transfer onto target lysine residues. Members of the cullin protein family are able to form cullin-based RING E3 ubiquitin ligases (CRLs) by binding to the small RING protein RBX1 that contact an E2 enzyme and simultaneously interacting with a substrate receptor (SR) module (Figure 1.2a). The substrate receptor module contains a small linker protein or domain with a substrate-specific adaptor to bring in substrates for ubiquitylation (Figure 1.2a). In human cells, there are seven cullin proteins (CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5 and CUL7) all capable of forming E3 ubiquitin ligases in the same complex architecture (Figure 1.2c). Together, CRLs regulate a spectacular array of cellular processes from DNA replication and mitosis to development and endocytosis.

The 3D structures of CRLs show their overall conformation to be an extended arc (Figure 1.2b). The cullin backbone consists of a helical N-terminal domain composed of three five-helix bundles, termed cullin repeats 1, 2 and 3, with a globular C-terminal domain (Figure 1.2b) (Angers et al., 2006; Zheng et al., 2002b). Linker proteins, or in the case of CUL3 substrate specific adaptors, contact cullins at their N-terminal region by interacting with helices 2 and 5, which are well conserved across different cullins (Angers et al., 2006; Zheng et al., 2002b). Interestingly, binding of linker protein DDB1 to CUL4A is additionally mediated by an extreme N-terminal
extension on the cullin which is wrapped around DDB1 (Figure 1.2b) (Angers et al., 2006). The C-terminal portion of cullins functions to bind the small RING protein RBX1 by formation of an intermolecular β-sheet through a winged-helix B (WHB) domain (Zheng et al., 2002b).

CRLs themselves have no enzymatic activity per se. The conformation of the cullin complex is such that substrate is sterically well-positioned for transfer of ubiquitin from the E2. However, for the formation of an isopeptide bond between the substrate’s lysine side chain and the carboxyl terminus of ubiquitin on the E2, the two must make physical contact. The substrate and RBX1 are bound at opposite ends of the cullin scaffold and the elongated structure of CRLs puts considerable distance between the two (Orlicky et al., 2003). Thus conformational flexibility is required to bridge this gap. After attachment of the first ubiquitin, elongation of a ubiquitin chain will likewise depend upon a CRL that’s able to change its conformation to accommodate the growing chain. This mechanistic mystery was solved when it was found that attachment of the ubiquitin-like protein NEDD8 (Neural precursor cell Expressed Developmentally Downregulated protein 8) to the C-terminal region of cullins induces conformational changes to stimulate ubiquitin transfer from the E2 to substrate lysine (Duda et al., 2008).

Cullin neddylation is carried out by an enzymatic cascade similar to that of ubiquitylation, requiring E1, E2 and E3 enzymes. The process of neddylation is indispensable for cell cycle progression, embryogenesis and viability. Upon NEDD8 attachment, drastic conformational changes occur in the C-terminal WHB domain of cullins, which partially releases RBX1 from this region to produce the flexibility needed to close the gap between the E2 bound to RBX1 and the substrate at the N-terminus of cullins, inducing ubiquitin transfer (Duda et al., 2008). In effect, the E2 bound to RBX1 is able
to swing back and forth not only to produce physical contact with the substrate lysine for initial ubiquitin transfer but also to allow for conjugation of more ubiquitin moieties. Through this mechanism, NEDD8 conjugation stimulates various aspects of CRL-mediated ubiquitylation from recruitment of the E2, to substrate ubiquitylation and polyubiquitin chain formation (Kawakami et al., 2013; Read et al., 2000). Disruption of the CUL1/RBX1 interaction by mutation of a single amino acid in the C-terminal part of CUL1 (Y761A) is enough to produce a constitutively active E3 ligase, further highlighting the significance of NEDD8-mediated regulation of CRL activity (Yamoah et al., 2008).

Cullin-based E3 ligases attach ubiquitylation modifications of various chain lengths and linkages to a vast number of substrates of different sizes, some of which contain multiple ubiquitylation sites. In order to integrate these mechanistic parameters, CRLs require extensive conformational variability and, in addition to neddylation, this is brought about by substrate adaptor-mediate dimerisation. CRL dimerisation provides two distinct distances between the E2 enzymes and substrate-binding sites, creating the possibility of accommodating different ubiquitin receptor sites as well as chain lengths and geometries (Tang et al., 2007). Additionally, bringing two E2 enzymes into proximity with the bound substrates increases the speed of ubiquitin transfer by essentially increasing the local concentration of reactants for ubiquitylation, leading to more efficient chain elongation (Varelas et al., 2003). Various CUL1 and CUL3 substrate adaptors have been found to function as dimers (Barbash et al., 2008; Chew et al., 2007; Stogios et al., 2006; Sumara et al., 2007; Tang et al., 2007). F-box proteins contain a hydrophobic D-domain of approximately 40 amino acids which mediate dimerisation (Tang et al., 2007). And similarly in BTB proteins, the contact surface allowing formation of CRL3 dimers is also hydrophobic (Stogios et
al., 2005). Reports of dimer formation in other cullins remain limited. The CRL2\textsuperscript{VHL} complex was reported to functionally dimerise (Chung et al., 2006). There is also some evidence pointing to dimerisation of CRL4s but specific examples of dimerising CUL4 adaptors and the protein domains required for dimer formation are not known (Chew et al., 2007).

Through the modular nature of CRLs, their assembly with different substrate adaptors directly control the target of ubiquitylation activity. Neddylation is essential for activating catalysis and in addition, CRL dimerisation is also required for the functionality of some cullin-based complexes. The assembly of CRLs, their neddylation and dimerisation together make up the basic CRL activation cycle. And this cycle is known to be regulated by two factors: the COP9 Signalosome (CSN) and CAND1. Current understanding is that the CSN and CAND1 regulate cycles of CRL neddylation and deneddylation and this enables efficient exchange of substrate receptor modules.

The CSN is an evolutionarily conserved complex composed of eight subunits (Wei et al., 1994). In the context of CRL regulation, the CSN acts to deneddylate and deubiquitylate the E3 ligases (Figure 1.3) (Cope and Deshaies, 2003). The CSN5 subunit is a zinc metalloisopeptidase and catalyses deneddylation of CRLs.

Early studies showed that inactivation of the CSN, while increasing the levels of neddylated cullins \textit{in vivo}, in fact led to reduced CRL activity presumably due to increased autoubiquitylation of substrate adaptors (Cope and Deshaies, 2006; Doronkin et al., 2003; Feng, 2003; Schwechheimer, 2001; Wee et al., 2005; Wu et al., 2005; Zhou et al., 2003). In addition, the deubiquitylase activity of the CSN complex was shown to be needed for stability of not only the SR module but also the cullin scaffold in
CRL1 and CRL3 complexes (Wee et al., 2005; Zhou et al., 2003). Thus, one function of the CSN is to protect CRLs from autoubiquitylation and losing their activity. Another proposed function of the CSN complex is that it binds and maintains the ligases in a “ready” state for substrate binding upon reception of an activating signal. This is exemplified by the rapid release of nucleotide excision repair complex CRL4^DDB2 from the CSN in response to UV irradiation (Groisman et al., 2003).

More recently studies of the CRL landscape in cells have revealed unexpected complexity in CSN-mediated regulation of cullins. In addition to CRL protection, the CSN is also thought to bind and thereby remove CRLs from the active pool. Biochemical studies have shown that presence of substrate is able to dissociate the CSN from CRLs (Emberley et al., 2012; Enchev et al., 2012). Also, quantitative mass spectrometry (MS) methods revealed that the CSN is able to bind both neddylated and deneddylated CRLs, and in fact a large proportion of neddylated cullins remains bound to the CSN in vivo (Bennett et al., 2010). Together, these findings indicate that substrate and CSN compete for binding of the CRL. As the concentration of substrate decreases due to ubiquitylation and degradation, the CSN is then able to bind to and remove the active neddylated CRL for deneddylation.

CAND1 is an evolutionarily conserved protein of 120kDa. It is able to bind the cullin scaffold by wrapping itself around the protein in a “head-to-tail” configuration to block both substrate adaptor binding and neddylation (Goldenberg et al., 2004; Liu et al., 2002; Zheng et al., 2002a). CAND1 is incapable of binding neddylated cullins; however, after deneddylation by the CSN, CAND1 can strip away the substrate recognition module (Figure 1.3) (Liu et al., 2002). In cells, a very small proportion of cullins are associated with CAND1 despite the availability of a free pool of the protein (Bennett et al., 2010). This suggests that cullin backbones only bind to CAND1 for a
limited period. The finding that both increasing and decreasing the interaction between CAND1 and CUL1 lead to reduced CRL activity shows that CAND1 functions to regulate a necessary assembly and disassembly cycle of CRLs (Zhang et al., 2008). Indeed, studies have shown that in vivo CAND1 promotes the activity of CRLs (Chuang, 2004). Recent in vitro studies show that CAND1 stimulates the exchange of SR modules and the physiological significance of this is demonstrated by the fact that the ablation of CAND1 activity leads to changes in the proportions of different SR modules bound to CUL1 (Pierce et al., 2013; Wu et al., 2013).

Together, the CSN and CAND1 regulate the CRL activation cycle, enabling efficient exchange between different substrate recognition modules (Figure 1.3). In light of the finding that the composition of the cellular CRL landscape is largely determined by the abundance of different SR modules, the combined activities of the CSN and CAND1 are postulated to ensure that the relative levels of SR modules is reflected in the composition of the CRLs in cells by promoting SR module exchange (Bennett et al., 2010).

Identified CRL substrates have been implicated in many cellular processes and their mode of recognition by the ligases have shown to be mechanistically varied. One prevailing theme among them is that they often require addition posttranslational modifications or an additional binding partner to associate with CRLs. Several F-box proteins recognise phosphodegrons on their cognate substrates and as will be discussed, CRL4CDT2 substrates require prerequisite binding to PCNA (proliferating cell nuclear antigen) for ubiquitylation (Abbas et al., 2010; Havens and Walter, 2009). An exotic example of substrate targeting is provided by CRL4DDB2, which is able to recognise and bind to UV-damaged DNA to target histones and a repair factor for ubiquitylation (Fischer et al., 2011). No doubt new CRL binding motifs will be discovered in future investigations.
In order to improve our understanding of CRLs and how they regulate our proteome, substrate identification of CRLs is critical. One limitation which has hindered substrate search via mass MS-based proteomics was the relative low abundance of ubiquitylated peptides. In recent years, a new method has emerged in CRL ubiquitylation substrate identification based on enrichment of ubiquitylated peptides to improve their detection by MS. As the last three amino acids of ubiquitin are arginine-glycine-glycine (RGG), tryptic peptides containing a ubiquitylated lysine will contain a di-glycine (di-gly or GG) ubiquitylation remnant. This new method relies on antibody-based enrichment of GG-peptides from ubiquitylated proteins (Xu et al., 2010). This has enabled large-scale screens to identify new degradation targets of CRLs by inhibition of the proteasome (Emanuele et al., 2011). It also allows more targeted approaches to determine the ubiquitylation substrates of individual CRLs via RNAi-mediated downregulation of the adaptor, which can then by confirmed in *in vitro* ubiquitylation assays and *in vivo* assessment of lysine mutants. This approach is expected to accelerate the process of novel substrate identification and in turn improve our understanding of CRLs.
1 Introduction

Figure 1.2 | The cullin-RING E3 ubiquitin ligases.

(a) Cullin RING E3 ubiquitin ligases have similar complex architectures. The adaptor confers specificity of ubiquitylation substrates and different modifications signal for degradation or changes to substrate activity, localisation or interaction partner. In the case of CUL3 complexes, the linker and adaptor are part of a single protein. (b) Structure of the DDB1-CUL4A-RBX1-SV5-V complex (Angers et al., 2006). DDB1 BPA, BPB and BPC stand for DDB1 β propeller A, B and C. (c) Complex architecture of CUL1-, CUL2/5-, CUL3- and CUL4A/b-based E3 ligases. For CRL1s, SKP1 is the linker protein and substrate specific adaptors are F-box proteins. CRL2s have ElonginC and ElonginB as linker proteins and adaptors are SOCS-box proteins. CUL3 directly interacts with substrates through BTB proteins and lastly CRL4A/B ligases bind different DCAFs through the linker DDB1 and DCAF adaptors recruit different substrates. The CRL-activating ubiquitin-like protein NEDD8 is also depicted.
The proposed CRL activation cycle is depicted. The exact mechanisms and stages of CRL activation cycle is still not well understood and remains an active area of research. The cullin backbone associates with the SR module upon synthesis and becomes neddylated which activates the CRL for substrate binding and ubiquitylation. The dimerisation of some CRLs have been shown to be important for their activities. As the concentration of substrate decreased due to polyubiquitylation and degradation, the CSN is able to bind the neddylated CRL and catalyse its deneddylation. This recycles the CRL and enables another round of activation. Alternatively, CAND1 binds the unneddylated CRL to displace the SR module and catalyse its exchange to reflect the abundance of SR modules in the cell. After SR module exchange the newly formed CRL can go through a new cycle of neddylation and substrate ubiquitylation. Diagram adapted (Lydeard et al., 2013).
1.1.3 CUL4-based E3 ubiquitin ligases

In human cells, Cullin 4 (CUL4) binds to DNA damage-binding protein 1 (DDB1) as the linker protein. Through modular associate to substrate adaptors (DCAF: DDB1 and CUL4 associated factors), Cullin 4-RING E3 ubiquitin ligases (CRL4s) regulate a range of activities in chromatin biology including DNA replication and DNA damage response, particularly to UV-induced lesions.

Mammalian cells express two closely related CUL4 paralogues CUL4A and CUL4B, both of which can assemble into CRLs which are structurally similar. The two paralogues have around 80% sequence identity where CUL4B contains an additional extreme N-terminal domain. There is evidence for an overlap in function between CUL4A and CUL4B, where both target substrates such as p21 and CDT1 for ubiquitylation and degradation (Jackson and Xiong, 2009; Lee and Zhou, 2007). However the two are not functionally redundant, demonstrated by the finding that mouse germline deletion of Cul4b led to embryonic lethality indicating a function of CUL4B that is not compensable by CUL4A. CUL4B has also been linked to X-linked mental retardation where CUL4B-deficient patients display various developmental defects (Zhao and Sun, 2012; Zou et al., 2007). Additionally, the two paralogues show differential regulation in their association with both the CSN and CAND1. A much larger proportion of CUL4B is associated with CAND1 in vivo than was found for CUL4A (E. Bennett, et al., 2010). However, the reason behind this observation and its significance are not understood.

The linker protein in CUL4 complexes, DDB1, is distinct from all other known cullin linkers. DDB1 is a large protein of 130kDa and X-ray crystallographic studies show that the protein contains multiple WD40 repeats that together fold into a triple β propeller configuration (Li et al.,
2006). Each β propeller contains seven WD40 repeats and the propellers are named BPA, BPB and BPC (β propeller A, B and C). Interestingly, the three β propellers are not formed in tandem from the amino acid sequence. Instead sequences from BPA and BPB are inserted into the sequence of BPC in formation of the domains. Structure of the CUL4-DDB1-RBX1 complex shows that BPB makes extensive contact with the N-terminal region of the CUL4A backbone. BPA and BPC together form a clam-like structure to present a binding pocket facing RBX1 at the C-terminus of CUL4 for substrate adaptors and other DDB1-interacting proteins (Angers et al., 2006). Indeed the structures of CRL4DDB2 and CRL4CSA ligases show that both adaptors insert a helix-loop-helix into the BPA-BPC double propeller binding pocket in order to bind the linker DDB1 (Fischer et al., 2011). Mutation studies have defined three clusters of exposed residues on BPC which different DCAFs use combinatorially to bind DDB1 (Jin et al., 2006).

Tandem affinity purifications (TAP) of DDB1 and CUL4 followed by mass spectrometry originally identified DCAFs as a family of substrate specific adaptors (Angers et al., 2006; He et al., 2006; Higa et al., 2006; Jin et al., 2006; Lee and Zhou, 2007). More than 60 unique DCAFs were identified in these effort and more than 50 of them contain WD40 repeats. Each WD40 repeat is composed of a four-stranded antiparallel β sheet. A single WD40 domain contains four or more WD40 repeats arranged around a central point forming a propeller-like structure. WD40 repeats in DCAFs only form one β propeller. Sequence analyses have revealed a relatively conserved ‘WDXR’ motif in DCAFs containing WD40 repeats located in linker regions between adjacent β strands. Some DCAFs were found to contain two or more such motifs and mutation studies have shown that they mediate binding to DDB1 (Angers et al., 2006; Jin et al., 2006). However WDXR motifs are not the only structures important for DDB1 association. The SV5 V protein does not
contain any WD40 repeats and was found to interact with DDB1 through the conserved surface residues on DDB1 BPC domain (Li et al., 2006). Furthermore, a helix-loop-helix domain found in many DCAFs, including DDB2 and CSA was also shown to be a key determinant required for DDB1 binding (Fischer et al., 2011).

Current evidence suggest multiple contact points and structural elements between DCAFs and DDB1. This mode of substrate adaptor recruitment by the linker protein is in stark contrast to the binding of adaptors in other cullins where simpler and more uniform strategies are employed. More mutagenesis and structural studies are needed to understand the DCAF-DDB1 binding strategy as well as to define the true set of DCAFs. Currently, a confident estimate of the number of DCAFs in human cells is around 50.

1.2 The DNA damage response and CUL4

1.2.1 The DNA damage checkpoint

DNA damage threatens the integrity of our genome. Ionising radiation causes DNA double-strand breaks (DSBs) which are highly toxic if left unrepaired. DNA damage can also take the form of alterations to one or both strands of the DNA duplex which block progression of the transcription machinery. By the same token, DNA replication can also become obstructed by lesions. This not only impedes the completion of genome duplication but also create local mechanical stress which can lead to replication fork collapse and the appearance of toxic DSBs.
The presence of DSBs leads to activation and phosphorylation of ATM (Figure 1.4). ATM then phosphorylates many targets and sets the stage for the formation of a damage foci where many proteins are recruited and modified including DNA repair proteins which clear away the damage. One target of ATM phosphorylation is histone H2AX on Ser139 to form γH2AX and this is used as a marker for DNA damage checkpoint activation. ATM also phosphorylates CHK2 which activates the p53 response leading to cell cycle arrest and apoptosis (Ciccia and Elledge, 2010; Wade and Elledge, 2007).

Endogenous and exogenous mutagens can cause chemical modifications or single-strand breaks in the DNA duplex. Some chemical alterations are present on one strand of the DNA only and others can create crosslinks between the two strands, termed interstrand crosslinks (ICLs). Lesions that block the DNA replication machinery causes replication stress that leads to activation of another branch of the DNA damage checkpoint (Figure 1.4) (Ciccia and Elledge, 2010; Wade and Elledge, 2007).

When there’s a blocking lesion on one strand of the DNA, DNA polymerase stalls at the lesion site. The activities of DNA polymerase and the MCM helicase becomes uncoupled and the helicase goes on to expose single-stranded DNA (ssDNA) (Byun, 2005). As a protective and signalling mechanism, RPA (replication protein A) binds to ssDNA, which activates another important DNA damage regulator, ATR (Zou and Elledge, 2003). ATR activates CHK1, triggering inhibitory tyrosine phosphorylation on cyclin-dependent kinase (CDK) and this leads to cell cycle delays at G1/S and during S phase (Costanzo et al., 2003). For the survival of the cell, it is imperative that DNA replication is completed during the time that is gained in delaying S phase progression. At stalled forks, lesion bypass is activated (Figure 1.4). This is achieved via either translesion synthesis or template switching signalled by PCNA mono- or poly-ubiquitylation respectively (Bienko et al.,
2010; Ciccia et al., 2012). Translesion synthesis involves recruitment of translesion synthesis polymerase which is able to replication over damage sites and template switching is the error-free bypass mechanism where a Holliday junction is establish and nascent DNA from the other strand is used as the template for replication to continue. (Bienko et al., 2010; Ciccia et al., 2012).

When two replication forks converge on an ICL site, where the two DNA strands are chemically linked, the Fanconi anaemia pathway is activated. Briefly, this repair pathway cleaves the replication fork at the ICL and coordinates repair activities of translesion synthesis, nucleotide excision repair and homologous recombination for recovery (Moldovan and D’Andrea, 2009).
Figure 1.4 | The DNA damage checkpoint.

DNA damage threatens the stability of our genome. A simplified version of the DNA damage response is depicted here. See text for more details. DSBs leads to activation of the ATM branch of the DNA damage checkpoint, this triggers the formation of a damage foci which activates damage repairs processes. ATM activation also leads to cell cycle arrest and apoptosis by signalling through CHK2 and p53. Replication stress occurs when replication forks are stalled at sites of DNA lesions, exposing ssDNA and activating the ATR branch of the DNA damage checkpoint. CHK1 is then activates which leads to cell cycle arrest. At sites of stalled replication forks, lesion bypass is also triggered.
1.2.2 Repair of DNA double-strand breaks

There are two major pathways which repair DSBs: non-homologous end joining (NHEJ), which re-ligates the broken strands together and is considered error-prone, and homologous recombination (HR), which uses newly replicated sister chromatid as a template for more accurate repair.

Canonical NHEJ is initiated when the Ku70/80 proteins sense DSBs and binds to the exposed DNA ends, which occurs within seconds (Mahaney et al., 2009). This is followed by recruitment and autophosphorylation of DNA-PKcs (DNA-dependent protein kinase catalytic subunit), which lead to activation of factors including the nuclease Artemis to trim DNA ends (Goodarzi et al., 2006). DNA ends are religated together by a DNA ligase complex that includes DNA ligase IV, XRCC4 (X-ray-cross-complementation group 4) and XLF (XRCC4 like factor). An alternative NHEJ pathway can also repair DSBs which is able to seal DNA ends independent of the canonical NHEJ factors (Frit et al., 2014).

After DNA replication has started and sister chromatid is synthesised, the HR repair pathway can be activated for repair of DSBs, which can result from for instance replication fork collapse. Since HR requires presence of the template for repair, the pathway is observed to be in its highest activity during late S and G2 phase when the most sister chromatids are available (Barlow et al., 2008). The HR pathway is depicted in Figure 1.5.

HR repair begins with 5’ end resection to generate an extended 3’ ssDNA overhang, which becomes coated by RPA (Figure 1.5) (Wold, 1997). This serves to protect the DNA and also to prevent secondary structures from
forming. RPA is subsequently displaced by RAD51 to form a nucleoprotein filament that catalyses homology search and formation of a D-loop (Figure 1.5). Synthesis and repair of the DSB can then ensue followed by D-loop cleavage. Alternatively, the other 3’ DNA overhang from resection of the opposite end of the DSB can be captured to form a Holliday junction (HJ). HJs can either be cleaved or dissolved to generate non-crossover or crossover products. Another branch of the pathway is synthesis-dependent strand annealing (SDSA), which does not involve HJ formation and results only in non-crossover products (Mehta and Haber, 2014).

The MRN (MRE11-RAD50-NBS1) complex is the initiator of HR (Figure 1.5). It is capable of sensing and signalling for DSBs by recruiting and activating pivotal DNA damage factors such as ATM and 53BP1 (p53-binding protein 1). ATM activates the DNA damage response, which can induce cell cycle arrest and apoptosis depending on the severity of damage occurred (Lee and Paull, 2004; Lee et al., 2009; Uziel et al., 2003). The MRN complex is also required to initiate DNA end resection, the first critical step of HR. The MRE11 nuclease in the complex binds to DSBs and starts initial DSB resection by first introducing a DNA single-strand nick via its endonucleolytic activity and subsequently exposing a limited 3’ overhang by digesting in a 3’ to 5’ direction (Shibata et al., 2014; Williams et al., 2007). The NBS1 subunit of the complex plays a regulatory role and is needed for localisation of the complex to sites of DNA damage (Williams et al., 2009). RAD50 contains a long coiled-coil domain through which two RAD50 proteins can dimerise (Lichten, 2005; van Noort et al., 2003). RAD50 binds to DNA and utilises its coiled-coils to tether the ends of DSBs, which is also vital for the MRN complex function (Lichten, 2005). Another early factor in HR repair is CtIP, the expression of which is cell cycle restricted to S and G2 phase (Limbo et al., 2007; Takeda et al., 2007). CtIP recruitment and
activating phosphorylation are regulated by BRCA1, an important HR boosting factor, and ATM respectively (Bunting et al., 2010; You and Bailis, 2010). Once at sites of DSBs, CtIP together with the MRN complex are both required to carry out initial end resection, a necessary step to initiate HR (Sartori et al., 2007).

The next stage of HR is extensive DNA end resection catalysed by more processive nucleases (Figure 1.5). To generate long 3’ ssDNA tails that become bound by RPA, the activities of EXO1 exonuclease and DNA2 helicase/endonuclease are required (Figure 1.5) (Mimitou and Symington, 2009). In addition, to aid resection and facilitate access by DNA repair factors, chromatin remodelling is needed to overcome the natural barrier of the chromatin structure. In this regard several chromatin remodelling proteins have been identified to function in DNA repair (Morrison et al., 2004; Tsukuda et al., 2005).

After exposed ssDNA is sequestered by RPA, its replacement by RAD51 to form nucleoprotein filaments is under the regulation of several proteins. HR mediators such as BRCA2, RAD52 and RAD51 paralogues stimulate formation of the filaments as well as strand invasion (Masson et al., 2001; New et al., 1998; Yang et al., 2005). The binding affinity of RPA for ssDNA is stronger than RAD51, which necessitates the activity of positive mediators of RAD51 filament formation (Sigurdsson et al., 2001b). The functions of these mediators are required for DSB repair by HR and genome stability as RAD51 filament formation and strand exchange are essential steps in HR repair of DSBs. Formation of RPA-coated ssDNA after resection also leads to ATR and CHK1 activation and CHK1-dependent phosphorylation of RAD51 has also been shown to be important for RAD51 recruitment to sites of damage (Sørensen et al., 2005).
Importantly, RAD51 filament formation is also negatively regulated. Wrongly triggered recombination can interfere with replication and needs to be attenuated. Also, disruption of RAD52 binding is required to allow DNA synthesis for repair, resolution and re-assembly of chromatin structure. In yeast, the Srs2 helicase was identified to counteract the function of RAD51, where addition of catalytic amounts of Srs2 was sufficient to disrupt RAD51 filament formation and subsequent recombination activity \textit{in vitro} (Krejci et al., 2003; Marini and Krejci, 2010). Although there is no report of a human Srs2 homologue, other helicases have been identified to perform a similar function, including BLM, RecQ5 and FANCJ (Bugreev et al., 2007; Hu et al., 2007; Sommers et al., 2009).

After formation of a D-loop following strand invasion and removal of RAD51, the 3’ end of the invading strand can then be extended by DNA polymerases before being re-ligated with the processed other end of the DNA break. This type of HR is SDSA and is thought to be promoted by the RTEL helicase (Barber et al., 2008). The D-loop can also be cleaved by MUS81/EME1 after repair to produce a crossover product. From a D-loop, a Holliday junction can be formed, which is a mobile junction between four strands of DNA. After repair the Holliday junction can either by dissolved non-nucleolytically, resulting in exclusively non-crossover events, or cleaved to produce either type of products.

In proliferating cells, dissolution is the primary mechanism for HJ processing and this is catalysed by the BTR complex (Manthei and Keck, 2013). This complex contains the BLM helicase, which is capable of unwinding multiple DNA structure (Ellis et al., 1995). Other factors in the complex include TopoII\textalpha, RMI1 and RMI2 (RecQ-mediated genome instability protein 1 and 2). TopoII\textalpha is a topoisomerase that function to relieve torsional stress, an activity required for proper HJ dissolution. RMI1
and RMI2 are both regulatory factors, which function to stimulate the activity of the complex and are required for complex stability (Ellis et al., 1995; Singh et al., 2008; Yin et al., 2005). The importance of accurate dissolution of HJs is highlighted by mutations in the BLM gene which lead to Bloom syndrome characterised by increased frequency of sister chromatid exchanges (SCEs). Similarly, disruption of TopoIIIα binding to BLM also leads to increased SCEs (Raynard et al., 2006). Another mechanism of processing HJs is through nucleolytic cleavage. MUS81/EME1, GEN1 and SLX1 are three known endonucleases which carry out cleavage resolution of HJs which results in both crossover and non-crossover (Chan and West, 2014; Gaillar et al., 2003; Rass et al., 2010; Svendsen et al., 2009).

The choice of DSB repair pathway between NHEJ and HR is under active regulation and current understanding is that this is predominantly focussed on regulation of DNA resection. The activities of 53BP1 and BRCA1 antagonise one another to stimulate either NHEJ or HR respectively (Figure 1.5). Upon appearance of DSBs 53BP1 localises to sites of damage acting to block 5’ end resection and promote NHEJ by facilitating DSB ends to find each other (Bunting et al., 2010; Dimitrova et al., 2008). In recent years, two proteins associated with 53BP1 were identified as potential effectors to inhibit resection in mammalian cells: Rif1 (RAP1-interacting factor 1) and PTIP (Pax2 transactivation domain interaction protein) (Gong et al., 2009; Zimmermann et al., 2014)(Figure 1.5). Rif1 is recruited to sites of DSBs and loss of Rif1 leads to increased DNA resection (Feng et al., 2013; Zimmermann et al., 2014). PTIP is required for 53BP1 localisation to damage sites and its ablation completely rescues HR in BRCA1-deficient cells (Callén et al., 2013; Wu et al., 2009). These results suggest that Rif1 and PTIP function with 53BP1 to inhibit end resection and may contribute to regulation of NHEJ and HR repair pathway choice (Daley and Sung, 2014; Gong et al., 2009;
Zimmermann et al., 2014). BRCA1 on the other hand promotes resection possibly through triggering removal of 53BP1 during S and G2 phase (Daley and Sung, 2014).
In S and G2 phase of the cell cycle HR is active. First, there is limited resection of the DSB ends mediated by MRN and CtIP where CtIP is activated by ATM. Extensive resection is carried out by EXO1 nuclease and DNA2 helicase. Exposed ssDNA is bound by RPA, activating ATR and CHK1. BRCA2 RAD52 and RAD51 paralogues stimulate RAD51 filament formation and strand invasion. RAD51 recruitment is also activated by active CHK1. After D-loop formation, repair occurs which may involve the formation of an HJ. SDSA leads to non-crossover. The D-loop can be cleaved leading to crossover. The HJ is either cleaved or dissolved resulting in crossover or non-crossover products. DNA resection is a point of regulation in HR versus NHEJ pathway choice, where 53BP1 with Rif1 and PTIP counteracts BRCA1. RTEL helicase promotes SDSA. MUS81/EME1 catalyse D-loop cleavage. HJ can be cleaved by MUS81/EME1, GEN1 and SLX1. HJ dissolution is carried out by the BTR complex. Red dotted lines and arrows show inhibition and activation.

Figure 1.5 | Homologous recombination repair of DSBs.
1.2.2 CUL4 and the DNA damage response

CUL4s have emerged as important regulators of many cellular processes including development, DNA replication and DNA damage repair (Jackson and Xiong, 2009). Loss of function of CUL4 in yeast or mammalian cells causes pleiotropic effects, many of which related to chromatin dysfunction. Furthermore, overexpression of CUL4A has been reported in several cancers (Lee and Zhou, 2010). In this section some important examples of CRL4-mediated regulation of DNA replication and DNA damage repair will be discussed.

In *C. elegans* *cul4* RNAi leads to massive DNA re-replication demonstrating the vital role of CUL4 in maintaining genome stability (Zhong et al., 2003). This is at least in part achieved through CUL4-mediated degradation of the replication licensing factor CDT1 and the CDK inhibitor p21 (Kim et al., 2008; Zhong et al., 2003). CDT1 and p21 degradation have been found in several model systems to be mediated through the substrate-specific adapter CDT2, which associates with CUL4 in a DDB1-dependent manner (Higa et al., 2006; Jin et al., 2006; Ralph et al., 2006; Sansam et al., 2006). Subsequently, another CRL4CDT2 substrate, SET8, was identified (Abbas et al., 2010). SET8 is a cell cycle-regulated protein and catalyses the monomethylation of histone H4 on lysine 20. This modification is important for many chromatin processes including regulation of DNA damage checkpoint and cell cycle progression (Julien and Herr, 2004; Sanders et al., 2004). CRL4CDT2 was found to mediate cell cycle regulation of SET8 levels via degradation, and loss of CUL4-dependent degradation of SET8 resulted in multiple defects including appearance of DNA damage and delayed cell cycle progression (Abbas et al., 2010).
Importantly, the degradation of CDT1, p21 and SET8 by CRL4CDT2 all depend on substrate binding to PCNA (proliferating cell nuclear antigen) via a PIP-box (PCNA-interacting peptides box) present in all three target substrates (Abbas et al., 2010; Havens and Walter, 2009; Kim et al., 2008).

In the context of genome replication CUL4 performs another essential function in nucleosome assembly after DNA replication. CUL4/DDB1, and their orthologues in yeast Rtt101/Mms1, were found to bind and ubiquitylate nascent acetylated histone H3 to promote transfer of histone H3-H4 dimers from chaperone Asf1 to other chaperones for nucleosome assembly. The H3-H4 dimer binds Asf1 with high affinity and ubiquitylation of H3 by CUL4/DDB1 weakens the affinity between Asf1 and H3 to facilitate dissociation (Han et al., 2013). Overexpression of Asf1a has been linked to poor prognosis in breast cancer indicating that dysregulation of nucleosome assembly contributes to carcinogenesis (Corpet et al., 2010).

Exposure to ultraviolet (UV) radiation can lead to the formation of toxic DNA lesions where neighbouring pyrimidine nucleotides become crosslinked creating pyrimidine dimers. These defects can block transcription. And during S phase the progression of DNA replication is also disrupted by these lesions and require error-prone translesion synthesis, which can introduce mutations. The nucleotide excision repair (NER) pathway is responsible for photolesion repair.

Two CRL4s, CRL4DDB2 and CRL4CSA, function in the two branches of NER: global genome NER (GG NER) and transcription-coupled NER (TC NER) respectively. The GG NER pathway surveys the whole genome for lesions and upon damage detection the NER complex is assembled and repair process begins (Volker et al., 2001). In this pathway, CRL4DB2 bind to and recognises photolesions and ubiquitylates histones and repair factor XPC to initiate NER.
repair complex formation (Guerrero-Santoro et al., 2008; Sugasawa et al., 2005; Fischer et al., 2011; Kapetanaki et al., 2006; Wang et al., 2006). CRL4<sup>CSA</sup>, which functions in TC NER, is recruited to sites of RNA polymerase II stalling and is involved in repair and transcription restart (Tornaletti, 2009). One ubiquitylation target of the complex is CSB, which is an early TC NER factor that binds to DNA at transcription block sites and recruits the rest of the repair complex (Troelstra et al., 2003). CRL4<sup>CSA</sup>-mediated degradation of CSB is essential for transcription recovery after repair (Troelstra et al., 2003).

1.3 S phase and histone production

1.3.1 Histone production

An essential process during DNA replication in S phase is the packaging of DNA into chromosomes once nascent DNA has been synthesised. DNA is first wrapped around the octomeric core histone complex comprising two of each of H2A, H2B, H3 and H4 to form individual nucleosomes. H1 is then needed to organise and compact the nucleosomes into higher order structures. In mammals, the genes which encode the histones exist in multiple copies and large gene families (Marzluff et al., 2002). The histones which are needed to package newly synthesised DNA are only produced during S phase and are thus referred to as replication-dependent histone proteins. They will from this point on be simply referred to as histone proteins.

The expression of histone genes are tightly cell cycle regulated with histone mRNAs increasing by around 35-fold upon S phase entry and again decreasing to basal levels at the end of DNA replication (Marzluff and Duronio, 2002). At the level of transcription, histone expression is regulated by Cyclin E-CDK2 target NPAT, which activates histone gene transcription at
the beginning of S phase (Liu et al., 2000; Ma et al., 2000). Maintaining correct regulation of histone production to meet the requirements of a proliferating cell is key to normal cell growth. Imbalances in histone production can compromise genome stability, disturb gene expression and have detrimental consequences for animal development (Kim et al., 1988; Meeks-Wagner and Hartwell, 1986; Pettitt et al., 2002; Prado and Aguilera, 2005).

Histone pre-mRNAs do not contain any introns and are only subjected to a single endonucleolytic cleavage in the nucleus at their 3' end to form mature mRNAs (Figure 1.6a) (Dominski and Marzluff, 1999). They are also the only eukaryotic mRNAs which lack a poly(A)-tail. All histone transcripts have a conserved stem-loop structure at their 3' end (Figure 1.6a), which is bound by the stem-loop binding protein (SLBP) (Wang et al., 1996; Dominski and Marzluff, 1999). SLBP is an important regulator of histone mRNA processing and protein expression, and it remains bound to the mRNA travelling with the transcript until the end of translation and mRNA decay is initiated. The expression of SLBP and histone mRNAs follow the same pattern, both are upregulated in S phase, and SLBP coordinates the accumulation of histone mRNA with the cell cycle (Sullivan et al., 2001). The cleavage site of histone mRNAs lies just 3' of the stem-loop and occurs after an ACCA sequence (Figure 1.6a) (Marzluff et al., 2008). Downstream of this site is the histone downstream element (HDE) which base-pairs with U7 snRNA in the U7 snRNP complex (Figure 1.6a,b) (Marzluff et al., 2008). Further characterisations carried out in recent years have revealed that the histone mRNA processing machinery shares several components with that of poly(A) mRNAs.

The processing of histone mRNA is depicted in Figure 1.6b. After the histone pre-mRNA is produced, SLBP binds to the stem-loop structure. Next,
the U7 snRNP which includes the U7 snRNA and a Sm/Lsm protein complex is recruited (Pillai et al., 2001). The Sm/Lsm protein complex contains the U7-specific proteins Lsm10 and 11 as well as the Sm B, D3, E, F and G proteins also found in spliceosomal snRNAs (Dominski et al., 2001b; Pillai et al., 2001). Binding specificity of U7 snRNP is conferred by base-pairing between the HDE and the U7 snRNA. SLBP and the zinc finger protein ZFP100 both increase the association affinity of the U7 RNP with histone pre-mRNA (Dominski et al., 2001b).

The catalytic components of histone mRNA processing are recruited to and positioned on the pre-mRNA by U7 snRNP and SLBP. The recruited components include the scaffolding protein Symplekin, the cleavage and polyadenylation specificity factor complex (CPSF, including CPSF30, CPSF73, CPSF100, CPSF160 and hFip1) and two proteins of the cleavage stimulatory factor complex (CstF, including CstF64 and CstF77) (Keller and Minvielle-Sebastia, 1997; Keon et al., 1996; Kolev, 2005; Takagaki and Manley, 2000). Both the CPSF and CstF are components involved in poly(A) site cleavage. The endonuclease CPSF73 is responsible for enzymatic cleavage of histone mRNA 3’ end, the same that mediates poly(A) site cleavage (Dominski et al., 2005). In recent years, the U2 snRNP, which was thought to be only involved in pre-mRNA splicing, was also identified to be recruited to histone mRNAs and contribute to efficient histone mRNA 3’ end cleavage (Friend et al., 2007). The recruitment of U2 snRNP to histone mRNA is achieved by recognition of a sequence element upstream of the stem-loop (Friend et al., 2007).

Once this large catalytically active complex has been assembled, the cleave can finally occur (Figure 1.6b). After 3’ end cleavage only SLBP remains associated with the processed histone mRNA. There would be considerable remodelling of the histone mRNP complex needed to remove
complexes which are no longer needed before nuclear export into the cytoplasm for translation.

For the translation of poly(A) transcripts, the mRNA adopts a ‘closed-loop’ conformation which protects it from decay and increase translation efficiency. This is also the case for histone mRNAs. The translation initiation factors eIF4E and eIF4G both bind to the 5’ cap of histone mRNA (Figure 1.7 bottom right). This 5’ end is directly tethered to the 3’ end SLBP-bound stem-loop element via SLBP Interacting Protein 1 (SLIP1), which interacts with both SLBP and eIF4G (Figure 1.7 bottom right) (Cakmakci et al., 2008). Additionally, SLBP directly interacts with the translation initiation factor eIF3, a protein of the 43S pre-initiation complex, to further stimulate translation (Figure 1.7 bottom right) (Gorgonia et al., 2005; Sanchez and Marzluff, 2002).

At the end of S phase histone mRNA and SLBP are both rapidly degraded. This is thought to be induced by Pin1-mediated proline isomerisation of SLBP to trigger its removal from the stem-loop (Krishnan et al., 2012; Zhang et al., 2012). The protein then shuttles to the nucleus where a phosphodegron regulates its polyubiquitylation and degradation (Krishnan et al., 2012). After SLBP dissociation, there is then addition of an oligo(U) tail by 3’ terminal uridyl transferases (TUTase) to the 3’ end of free histone mRNA (Mullen and Marzluff, 2008). This tail is bound by a complex of Lsm 1-7 proteins which stimulate 5’-end decapping followed by 5’ to 3’ exonucleolytic degradation. 3’ to 5’ degradation has also been shown to occur (Mullen and Marzluff, 2008).
Figure 1.6 | Histone mRNA 3’ end cleavage.

(a) Histone mRNA processing involves a single cleavage adjacent to a conserved stem-loop. (b) SLBP binds to the stem-loop and the U7 snRNP is recruited to the mRNA via base-pairing with the HDE and protein-protein interactions. A large number of proteins and protein complexes (symplekin, CstF, CPSF and the U2 snRNP) then assemble to catalyse the 3’ end cleavage, which is mediated by the endonucleolytic activity of CPSF73. The Lsm 10 and Lsm 11 are simply labelled as 10 and 11. The Sm proteins are B, D3, E, F and G. CPSF, cleavage polyadenylation specificity complex. CstF, cleavage stimulatory factor complex.
1.3.2 SLBP is central to histone expression

As can be appreciated in Figure 1.7, SLBP-bound stem-loop goes on to regulate every stage and aspect of histone mRNA expression. The importance of SLBP in histone mRNA processing is highlighted by the fact that SLBP knockdown leads to a 50% reduction in histone mRNAs in cells (Sullivan et al., 2009). This defect in histone mRNA processing and export inevitably leads to reduced levels of histone proteins associated with S phase delay and CHK1-mediated checkpoint activation (Sullivan et al., 2009).

After histone mRNA processing, SLBP remains bound to the mature transcript and is exported to the cytoplasm. Translation of the mRNA is also regulated by SLBP-bound stem-loop which is involved in circularising the transcript and stimulating translation (Sanchez and Marzluff, 2002). During S phase, SLBP is postulated to dislodge from the mRNA and shuttle back to the nucleus to activate processing of more histone mRNAs (Krishnan et al., 2012). Pin1-mediated proline isomerisation about a conserved phosphorylated Thr-Pro sequence is postulated to regulate dissociation of SLBP from the histone mRNA stem-loop (Krishnan et al., 2012; 2014; Zhang et al., 2012). SLBP alone is specifically localised to the nucleus only, and it is through association with histone mRNAs that the protein travels to the cytoplasm (Erkmann et al., 2005b). And finally the stability of histone mRNA is also co-regulated with that of SLBP where SLBP removal and proteolysis triggers mRNA degradation at the end of S phase. Thus the entire life cycle of histone mRNAs is critically dependent on SLBP.

SLBP is a relatively small protein (254 amino acids) (Figure 1.8). The N-terminal end of the protein consists of loops and is not well-structured. However, the crystal structure of its central and C-terminal portions bound to histone mRNA stem-loop and human 3′-5′ exonuclease 3′hExo has been
determined (Tan et al., 2013). The N-terminal region of the protein contains two nuclear localisation sequences required for nuclear targeting of the protein in S phase (Figure 1.8) (Erkmann et al., 2005a). This portion of the protein has also been shown to stimulate histone mRNA translation through interaction with SLIP1 (Cakmakci et al., 2008; Sanchez and Marzluff, 2002). The phosphodegron (amino acids 59 to 63) which triggers degradation of SLBP at the end of S phase also lies in the N-terminal region of SLBP (Zheng et al., 2003).

The middle and C-terminal half of the protein contain highly conserved amino acid sequences (Dominski et al., 2001a). This portion of the protein is required for histone mRNA stem-loop binding and efficient cleavage of the 3’ end for mRNA maturation (Figure 1.8) (Dominski et al., 2001a). Deletion studies have identified a unique RNA-binding domain (RBD) on SLBP 73 amino acids in length (aa 128 to 198), which is rather dissimilar to other known RNA-recognition motifs (RRM) (Dominski et al., 2001a). Although one commonality between the SLBP RBD and other RRMs is that both contain basic residues. These are usually highly conserved arginines and lysines which are important to establish electrostatic interactions with the phosphate groups of the recognised target RNA sequence. And lastly, the residue which undergoes proline isomerisation postulated to dissociate SLBP from histone mRNA is also in the RNA processing region of the protein. Upon phosphorylation-dependent proline isomerisation, the contact sites between the RBD and histone mRNA stem-loop is disrupted (Krishnan et al., 2012).
SLBP regulates the entire life cycle of histone mRNAs. It becomes bound to the stem-loop when histone mRNAs are produced and is required for efficient processing of the 3’ end. The U7 snRNP is an early factor which is recruited by binding to SLBP via ZFP100 and base pairing to the mRNA itself. Then a large number of proteins and complexes are recruited to histone mRNA and the 3’ end is formed via a single endonucleolytic cleavage mediated by CPSF73. Processed histone mRNA, along with SLBP are exported out of the nucleus for translation. SLBP, bound to the 3’ end of histone mRNA, is tethered to the 5’ cap directly, and indirectly via SLIP1, through binding to pre-initiation factors. This tethering stimulates histone mRNA translation and is crucial for efficient histone expression. 4E, eIF4E; 4G, eIF4G; 3, eIF3.

Figure 1.7 | Histone production in S phase.
Figure 1.8 | SLBP domain structure.

The N-terminal domain of SLBP is important for proper nuclear localisation of the protein and was found to be important for translation initiation of histone mRNAs. This portion of the protein also contains the phosphodegron which regulates SLBP degradation at the end of S phase. The C-terminal region of the protein contains the RNA-binding and RNA-processing domains which contact histone mRNA stem-loop. Phosphorylation of a specific threonine and isomerisation of the adjacent proline regulates SLBP dissociation from histone mRNA. SLBP dislodge from mRNA promotes its polyubiquitylation at the end of DNA replication and may allow nuclear shuttling.
1.4 Aim of this project

Although many putative CUL4 adaptors are known, their functions and more importantly their ubiquitylation substrates have remained uncharacterised. In order to identify novel CUL4 substrate adaptors that function in cell cycle progression, a live-cell imaging-based RNAi screen was performed prior to the start of this project (Piwko et al., 2010). Here, a HeLa cell line stably expressing EGFP-tagged PCNA was used to visualise the progression of interphase. The expression of EGFP-PCNA goes through characteristic morphological changes throughout G1, S and G2 phases allowing the duration of these phases to be determined.

The screen was carried out on a library of 147 genes which included putative CUL4 adaptors as well as CUL4-interacting proteins. Each protein was downregulated using between 3 and 6 different siRNAs in a 96-well plate format. Image acquisition commenced after 25 hours of knockdown for 48 hours with a time resolution of six minutes. The image analysis was performed in an automated fashion to yield a timeline of cell trajectories with the lengths of the different cell cycle phase. Using this approach, several genes were identified where their downregulation led to delays in G1/S or S phase. The main aim of this doctoral project is to confirm the hit proteins from the screen and further characterise a subset of the candidate adaptors.

After confirming and identifying the candidate adaptors to study further, the downregulation phenotypes of the adaptors in terms of DNA replication and DNA damage responses were characterised. This was done to establish a functional role for the E3 ligases in particular pathways within the broad processes of replication and DNA damage repair. Results from this part of the project was used to make inferences on possible ubiquitylation targets of the E3 ligases.
This project was broken down into three phases. Firstly, the list of identified adaptors from the screen was confirmed using complementary experimental methods and some adaptors were chosen as the final candidates for further characterisation. Next, detailed analysis of the downregulation phenotypes of candidate adaptors was carried out in order to determine the biological processes the adaptors function in. And ultimately, to arrive at a deeper understanding of the function of the CRL4s, efforts were made to identify the ubiquitylation substrate for one of the adaptors.

The goal of the project was to better understand how CUL4 exerts its function in maintaining normal S phase progression and DNA damage repair through characterisation of novel substrate-specific adaptors.
2 Results I: Candidate gene selection

The CUL4 substrate adaptors which gave the strongest G1 and S phase delay phenotypes as identified from the live-cell imaging-based RNAi screen included 13 proteins. This initial list of candidate genes were subjected to further experimental confirmation and final candidate selection.

Three assays were chosen to assess the downregulation phenotypes of the candidates in HeLa cells. Pools of 3 to 4 different siRNAs targeting each gene from the screen were used for RNAi-mediated downregulation in the three candidate selection assays. BrdU incorporation assay was performed to evaluate the DNA replication rate of cells. The cell cycle profiles of siRNA-treated cells were assayed via flow cytometry. The formation of γH2AX and 53BP1 foci was also probed by immunofluorescence microscopy to check the extent of DNA damage signalling in cells. In each experiment all quantifications from candidate gene knockdown were compared to that of CUL4 and/or DDB1 downregulation as positive controls and to examine the severity of the observed phenotypes.

In the BrdU incorporation assay, cells depleted of CUL4 showed more than 50% reduction in DNA replication rate (Figure 2.1). Downregulation of a number of candidate genes, including WDR23, WDTC1 and WDSOF1, also exhibited a reduction in DNA synthesis to a similar level. For WDR23, DCAF6 and WDSOF1 this reduction in DNA synthesis was also accompanied by
delays in S phase progression as determined in flow cytometry experiments (Figure 2.2). The cell cycle profiles of CUL4- and DDB1-depleted cells showed strong S and G2 phase delays respectively, both of which are previously well-characterised phenotypes for the two proteins (Figure 2.2). One cellular defect which can lead to cell cycle delays is the presence of DNA damage and DNA damage signalling. Thus, cells depleted of the candidate genes were also subjected to immunofluorescence staining to visualise the appearance of 53BP1 and γH2AX foci. Both being markers for DNA damage signalling (Figure 2.3). WDR23, WDR42a, DCAF6 and WDTC1 depletion all led to increased levels of γH2AX and 53BP1 foci at levels which were similar to or stronger than that of CUL4 downregulation.

In the automated bioinformatics analysis of image sequences from the screen, trajectories of cell division were followed. However, if a cell in the image sequence undergoes apoptosis, this particular trajectory stops being analysed and the information of how much apoptosis there is upon knockdown of this particular adaptor is lost. Because of this reason, adaptors which showed increased DNA damage foci staining were chosen for manual analysis of their raw imaging data. In the case of WDR42a re-examining the screen images by eye gave fruitful observations, where siRNA-mediated knockdown of the adaptor resulted in many cellular trajectories ending strikingly in apoptosis (Figure 2.4).

Based on the results of these further analyses, four final candidates were chosen which gave the strongest phenotypes in the selection assays described above compared to CUL4 and DDB1 depletion. These candidates are WDR23, WDTC1, WDSOF1 and WDR42a.
Results 1: Candidate gene selection

Figure 2.1 | BrdU incorporation of hits from the screen.

(a,b) Hits identified from the screen were depleted by RNAi in HeLa cells utilising a pool of siRNAs used for the screen. After 72 h of siRNA treatment, cells were pulsed with BrdU for 30 min prior to harvesting. Cells were fixed and stained with FITC-conjugated anti-BrdU antibody and assayed by flow cytometry. Quantifications were performed using FlowJo software and data in graph shows mean with SEM and was arranged in decreasing levels of BrdU incorporation. The dashed red line represent BrdU incorporation of CUL4 depletion.
Figure 2.2 | Cell cycle profiles of hits from the screen.

HeLa cells were treated with a pool of siRNAs targeting each of the hits from the screen for 72 h. Cells were harvested by trypsinisation and ethanol fixed. The DNA content was determined using propidium iodide staining and assayed by flow cytometry. Data analysis was done using FlowJo.
Figure 2.3 | Staining of DNA damage markers in hits from the screen.

(a,b) Hits from the screen were downregulated by RNAi in HeLa cells for 72 h. γH2AX and 53BP1 foci were visualised using specific antibodies in methanol fixed cells. DAPI was used for nuclear staining. Image analysis of was carried out in ImageJ. γH2AX-positive cells were defined as those containing ≥10 foci per cell. Data in graph shows mean with SEM. The dashed red line shows the percentage of γH2AX-positive cells upon CUL4 downregulation.
Figure 2.4 | WDR42a downregulation leads to excessive apoptosis.

Raw image sequence taken from the RNAi screen of WDR42a depletion (bottom) compared to control (top) cells. HeLa cells stably expressing replication marker EGFP-PCNA used in the RNAi screen were imaged for 48 h after 25 h of WDR42a depletion.
3 Results II: CRL4$^{\text{WDR23}}$ ubiquitylates SLBP for efficient histone mRNA processing

3.1 Introduction

During S phase, the entire genome of the cell needs to be accurately reproduced. Besides faithful replication of the DNA sequence itself by the replication machinery, chromatin structure has to be re-established in the nascent DNA duplex. Defective chromatin assembly after replication disrupts transfer of epigenetic information and threatens cellular genetic integrity (Alabert and Groth, 2012). Insufficient histone supply has been linked to accelerated ageing (Feser et al., 2010; O'Sullivan et al., 2010); and altered histone production and deposition are associated with developmental disorders (Ask et al., 2012; Kerzendorfer et al., 2012).

Histone mRNAs are unique in eukaryote. They are intron-less, contain a highly conserved stem-loop structure and are not polyadenylated. Instead a single 3’ end cleavage adjacent to the stem-loop is required for its maturation (Dominski and Marzluff, 1999). The stem-loop is bound by the stem-loop binding protein (SLBP), an important regulator of histone mRNA expression. It regulates the entire lifecycle of the transcripts including its 3’
end processing, translation and stability (Marzluff et al., 2008; Nicholson and Müller, 2008; Sullivan et al., 2001). A number of large mRNA processing protein and ribonucleoprotein complexes are recruited to carry out catalysis of 3’ end formation and several of these components are shared between processing of histone and poly(A) mRNA (Nicholson and Müller, 2008; Yang et al., 2012). After the nucleolytic cleavage, SLBP stays bound to the processed mRNA and are together exported to the cytoplasm for translation, where it also stimulates translation (Marzluff and Duronio, 2002; Gorgonia et al., 2005; Sanchez and Marzluff, 2002). At the end of S phase, SLBP is removed from the stem-loop, destabilising the transcripts, which are degraded by exonucleases (Mullen and Marzluff, 2008). SLBP itself is also degraded at the end of DNA replication (Zheng et al., 2003).

Cullin 4 (CUL4) RING E3 ubiquitin ligases (CRL4s) regulate important processes in chromatin biology including DNA replication and DNA damage responses (Jackson and Xiong, 2009). CRL4s, like other CRL complexes, are composed of the cullin backbone (CUL4A or CUL4B) which can interact with different substrate adaptors via a specific linker protein (DDB1) to bring in substrates for ubiquitylation. Although the role of CRL4s is strongly implicated in chromatin-related processes, only a handful of individual CRL4s are mechanistically well understood.

Various interaction and bioinformatics studies have shown that more than 50 CUL4 adaptors exist in human cells (Angers et al., 2006; Fischer et al., 2011; He et al., 2006; Higa et al., 2006; Jin et al., 2006; Lee and Zhou, 2007). In order to improve our understanding of CRL4s, our group carried out an RNAi live cell imaging screen on a library of CUL4 interactors and adaptors to study their roles in cell cycle progression (Piwko et al., 2010). From this approach, we identified a number of CUL4 adaptors where their RNAi-mediated downregulation led to delay in G1 or S phase. Amongst these
was the putative CUL4 adaptor WDR23. Here, we present functional characterisation of the CRL4$^{\text{WDR23}}$ E3 ubiquitin ligase where we identify a novel role for the E3 ligase in histone mRNA processing.

3.2 Results

3.2.1 WDR23 is required for normal DNA replication

To gain insights into the function of WDR23, we first investigated its downregulation phenotypes. Depletion of this CUL4 adaptor led to significantly reduced rates of cell growth (more than 70% reduction) in HeLa cells in clonogenic assays (Figure 3.1a,b). The RNAi screen identified WDR23 to be important for S phase progression. In double-thymidine block release experiments using U2OS cells, we confirmed that progression through S phase was strongly delayed upon WDR23 downregulation (Figure 3.1c). In the control, most cells had progressed into G2/M after 6 hours of release from thymidine block whereas in WDR23-downregulated cells, the majority of cells were still delayed in S phase after 9 hours of release (Figure 3.1c).

Next we specifically investigated the effects of WDR23 knockdown on DNA replication. We found that adaptor depletion led to severely reduced BrdU incorporation in U2OS cells, showing that DNA synthesis in S phase was inhibited (Figure 3.1d). We then assessed the progression of individual replication forks in DNA fibre assays and found that lengths of DNA fibre tracts were severely shortened upon WDR23 knockdown (Figure 3.1e,f; Figure 3.2). As can be appreciated in the quantifications of DNA fibre lengths, WDR23 depletion resulted in not only a severe but also a global slow down of replication fork speed (Figure 3.1f).
Immunoblot analysis of extracts derived from WDR23-depleted cells revealed an increase in phosphorylation of RPA2 and CHK1 S345 suggesting the presence of replication stress (Figure 3.1g). One downstream effect of CHK1-dependent checkpoint signalling is replication and S phase slowdown. This checkpoint-dependent S phase delay was confirmed by inhibition of ATR- and ATM-mediated checkpoint activation via caffeine treatment of WDR23-depleted cells. Upon treatment with caffeine, the S phase delay phenotype of WDR23 downregulation was seen to diminish (Figure 3.3).

Together, these results show that WDR23 is required for normal DNA synthesis. Upon its knockdown progression of replication forks is inhibited leading to strong checkpoint-dependent S phase delay and ultimately leads to impairs cell growth.
Figure 3.1 | WDR23 knockdown leads to DNA replication, cell cycle and growth defects.

(a,b) HeLa cells depleted of WDR23 were trypsinised and seeded in 6-well plates and allowed to grow for 7 days. Colonies were stained and assessed by quantifying integrated intensities using ImageJ. (c) WDR23 depletion was done in U2OS cells followed by double-thymidine block release. Samples were harvested at indicated time points after release and fixed for determination of DNA content by propodium iodide staining in flow cytometry. (d) WDR23 was downregulated in U2OS cells and pulsed with BrdU for 30 min prior to harvesting. Cells were fixed and stained with FITC-conjugated anti-BrdU antibody and assessed by flow cytometry. (e) CUL4 and WDR23 depletions were carried out in HeLa cells. Cells were then pulse labelled with CldU and IdU for 30 min each consecutively. DNA tracts were spread on glass slides after cell lysis and incorporated CldU and IdU were detected using specific antibodies against the nucleotides. (f) DNA fibre tract lengths (>130 tracts per sample) were measured in ImageJ and quantified in Matlab. (g) Downregulation of WDR23 was done in HeLa cells and phosphorylation of pRPA2 and CHK1 Ser345 were assessed by immunoblotting using specific antibodies. GAPDH included as control for loading.
Results II: CRL4WDR23 ubiquitylates SLBP for efficient histone mRNA processing

Figure 3.2 | WDR23 depletion leads to significantly shorter DNA fibres.

(a) In order to confirm only unbroken tracts were used in quantification of DNA tract lengths, the lengths of the two halves of all fibres, red CldU and green IdU tracts, were quantified. As cells were pulsed with CldU then IdU for the same amount of time, the two halves of all intact tracts should be equal in length, which is demonstrated in the graph. (b) Raw images of DNA fibres in cells depleted of CUL4 and WDR23 for 72 h.
3.2.2 Histone pool depletion causes DNA replication defects upon WDR23 knockdown

Since the major phenotype of WDR23 downregulation is a strong global inhibition of DNA replication, we next sought to determine the cause for this defect. An important requirement for DNA replication and normal S phase progression is a sufficient supply of histones to package newly synthesised...
DNA into nucleosomes and re-establish chromatin structure. Upon examination of the histone pool, we found by Coomassie staining that the protein levels of all five canonical histones were reduced upon prolonged WDR23 depletion and that this reduction progressively worsened with longer WDR23 siRNA treatment (Figure 3.4a). Next, the levels of total histone mRNAs were determined by qRT-PCR and a significant reduction in the levels of histone transcripts was observed upon adaptor depletion in HeLa and U2OS cells (Figure 3.4c; Figure 3.5). This suggests that a lack of available histone mRNA is leading to a reduction in histone proteins.

To produce mature histone mRNAs, the pre-mRNA is subjected to a 3’ end cleavage. So we next probed the relative levels of un-cleaved histone mRNAs to deduce whether a reduction in histone transcripts could be due to suppressed 3’ end cleavage. Specific qRT-PCR primers were designed which amplify across the cleavage site in the pre-mRNA and these were used to quantify the levels of uncleaved transcripts in cells (Figure 3.4b). Upon WDR23 downregulation uncleaved histone transcript levels were strongly accumulated (Figure 3.4d; Figure 3.5)

In order to confirm that the histone mRNA processing defects are WDR23 specific and not due to off-target effects of RNAi, phenotype-complementation assays were carried out by overexpression of an RNAi-resistant WDR23 variant. The RNAi-resistant WDR23 construct was created by introducing silent mutations into the target sequence of the WDR23 siRNA. For the complementation assays, this construct was then stably expressed from a doxycycline-inducible promoter. We found that expression of exogenous RNAi-resistant WDR23 partly alleviated the aberrant accumulation of uncleaved histone mRNAs and concomitantly restored the levels of mature histone mRNAs (Figure 3.6 a,b,c). This partial rescue of histone mRNA processing defects was accompanied by similar restoration of
normal DNA replication, cell cycle progression and growth as assessed by BrdU incorporation, flow cytometry and clonogenic assays (Figure 3.6 d,e; Figure 3.7; Figure 3.8).

Taken together these results indicate WDR23 is required for efficient 3’ end cleavage of histone mRNAs for histone production and its depletion leads to reduced histone supply during S phase which inhibits DNA replication and cell growth.
Figure 3.4 | WDR23 downregulation leads to histone pool protein and mRNA depletion.

(a) Cell extracts were made from HeLa cells depleted of WDR23 for 96 h and 120 h. Proteins were separated by SDS-PAGE and visualised by Coomassie staining. Bands corresponding to canonical histones are indicated. WDR23 and GAPDH were immunoblotted using specific antibodies to assess downregulation efficiency and equal loading respectively. (b) Illustration shows basic structure of canonical histone mRNAs including the histone ORF (open reading frame), stem-loop, cleavage site sequence and the HDE. The red arrow (bottom) marks position of the 3’ end cleavage to produce mature histone mRNAs and the blue arrows show positions of qRT-PCR primers used to assess amount of mature (top) and 3’ uncleaved (bottom) histone mRNAs. Note: normally, the levels of un-cleaved histone mRNAs are low in cells. So although the primer pair used for assessment of mature histone mRNAs will amplify both species, the majority of mRNAs amplified are mature processed mRNAs. (c) qRT-PCR analysis of mature histone mRNA in HeLa cells depleted of WDR23 for 72 h. (d) qRT-PCR analysis of 3’ uncleaved histone mRNAs in HeLa cells depleted of WDR23 for 72 h. Data in qRT-PCR graphs (c) and (d) are relative to control cells and show mean across independent experiments with technical triplicates and SEM.
Results II: CRL4WDR23 ubiquitylates SLBP for efficient histone mRNA processing

Figure 3.5 | WDR23 depletion leads to histone mRNA processing defects in U2OS cells.

Cells were depleted of WDR23 for 72 h and relative processed and uncleaved histone mRNAs were quantified by qRT-PCR. The graph shows mean across independent experiments with technical triplicates. Error bars show SEM.
Results II: CRL4WDR23 ubiquylates SLBP for efficient histone mRNA processing

Figure 3.6 | WDR23 knockdown phenotypes are rescued by expression of RNAi-resistant WDR23 variant.

Analysis of ability to rescue WDR23 downregulation phenotypes by RNAi-resistant WDR23 variant in HEK 293 cells. Depletion of WDR23 was performed for 72 h and expression of RNAi-resistant WDR23 variant was induced by doxycycline addition at the time of downregulation. (a) Expression of RNAi-resistant WDR23 variant was assessed by immunoblotting using specific WDR23 antibody. GAPDH was included as a loading control. (b,c) Levels of (b) uncleaved and (c) mature histone mRNAs were quantified using qRT-PCR. Graphs show mean across independent experiments with technical triplicates relative to control with SEM. (d) Cells were pulsed with BrdU for 30mins before being fixed and stained with FITC-conjugated anti-BrdU antibody and analysed by flow cytometry. DNA was stained with propodium idodie. (e) To assess growth and survival cells were trypsinised and seeded into 6-well plates and allowed to grow for 7 days. Colonies were stained.
Figure 3.7 | Expression of RNAi-resistant WDR23 variant rescues replication and growth defects in WDR23-depleted cells.

Depletion of WDR23 was performed for 72 h and expression of RNAi-resistant WDR23 variant was induced by doxycycline addition at the time of downregulation. (a) Replication was assessed by BrdU incorporation. Cells pulsed with BrdU were assayed by flow cytometry using FITC-conjugated anti-BrdU. Quantifications were done in MATLAB. (b) Cell growth was assessed in clonogenic assays. After colonies were stained, ImageJ was used to quantify integrated intensity and this used as an indication of colony formation. Graphs show mean with SEM.
Figure 3.8 | Rescue of cell cycle defects by exogenous expression of RNAi-resistant WDR23 is dependent on formation of CRL4<sup>WDR23</sup> active ligase and ubiquitylation.

HEK 293 cells were treated with control versus WDR23 siRNA for 72 h with and without either full length WDR23 (FL) or N-terminal deletion WDR23 mutant (ΔN) exogenous expression. Exogenous expression of WDR23 FL and ΔN were induced 12 h after RNAi treatment. Cells were fixed and DNA content assessed by propidium iodide staining and flow cytometry.
3.2.3 The function of WDR23 in histone mRNA processing is dependent on CUL4 and ubiquitylation

WDR23 forms an active E3 ubiquitin ligase, so we next examined whether its novel function in histone mRNA processing is dependent on complex formation with DDB1/CUL4 and ubiquitylation. WDR23 contains a small helix-loop-helix (HLH) domain which was demonstrated to be important for binding to DDB1 and formation of a CRL4 complex (Fischer et al., 2011). We constructed a mutant lacking this HLH domain (WDR23ΔN) and we found that this mutant does not bind DDB1/CUL4 (Figure 3.9a), and therefore should not be able to form an active E3 ubiquitin ligase. This mutant was then used to assess the CUL4 and ubiquitylation dependence of observed WDR23 phenotypes.

Acting in a dominant-negative manner, exogenous expression of WDR23-ΔN mutant in RNAi control cells mimicked the histone mRNA processing defects seen in cells depleted of WDR23, where uncleaved mRNA accumulated and processed transcripts were reduced (Figure 3.9c,d). The cell cycle profile and growth rates were also determined by flow cytometry and clonogenic assays respectively. Expression of WDR23-ΔN in control cells and downregulation of WDR23 both resulted in S phase delay and grossly impaired growth (Figure 3.9e and Figure 3.8). On the other hand, overexpression of full length WDR23 in cells treated with control siRNA did not result in histone mRNA processing or cell cycle defects (Figure 3.8, Figure 3.10).

These data show that the function of CUL4 adaptor WDR23 in histone mRNA processing requires the formation of an active CUL4 E3 ubiquitin ligase and suggest this function also depends on ubiquitylation.
Figure 3.9 | WDR23 function in histone mRNA processing requires CRL4<sup>WDR23</sup> ligase formation.

(a) WDR23-SSH (SSH, strep-strep-HA tag) and WDR23ΔN-SSH mutant were immunoprecipitated from extracts prepared from stably-expressing HeLa cells using anti-HA antibodies. The immunoprecipitates were immunoblotted for the presence of DDB1 and CUL4A using specific antibodies. (b) Extracts prepared from cells exogenously overexpressing WDR23ΔN were made and proteins separated by SDS-PAGE. HA-tag was immunoblotted to confirm expression of WDR23ΔN. WDR23 was immunoblotted using specific antibody to assess downregulation efficiency. GAPDH was included as loading control. (c,d) 3’ uncleaved and processed histone H1 mRNAs were analysed by qRT-PCR in HeLa cells treated with control siRNA, with and without WDR23ΔN exogenous expression, versus WDR23 downregulation for 72 h. Exogenous expression of WDR23 ΔN was done 12 h after RNAi treatment. (e) Same treatment conditions were done as in (c,d). Cells were seeded into 6-well plates and incubated for 7 days. Colonies were stained. Colony formation was assessed by quantifying integrated intensities using ImageJ. Data in graphs show mean across independent experiments with technical triplicates and SEM. qRT-PCR graphs depict relative levels with respect to control.
Results II: CRL4WDR23 ubiquitylates SLBP for efficient histone mRNA processing

3.2.4 SLBP interacts with WDR23 and is a ubiquitylation substrate of CRL4\textsuperscript{WDR23}

SLBP is an important factor required for efficient processing and translation of histone mRNAs. Downregulation of SLBP resulted in an accumulation of uncleaved histone mRNAs similar to that observed for WDR23 depletion (Figure 3.11), and this led us to study the functional relationship between the two proteins. Intriguingly, WDR23 knockdown led to an observed mislocalisation defect in SLBP (Figure 3.12). Upon adaptor depletion, the predominantly nuclear localisation pattern of SLBP became...
largely cytoplasmic (Figure 3.12), suggesting a functional interaction between the two proteins. Additionally, we found similar expression patterns for the two proteins where both proteins are upregulated upon S phase entry (Figure 3.13) (Whitfield et al., 2000). We next stably expressed strep-strep-HA tagged WDR23 and found that SLBP co-immunoprecipitated with WDR23 along with DDB1 and CUL4 (Figure 3.14a). We then immunoprecipitated endogenous SLBP using an antibody against the protein and found WDR23 and DDB1 specifically co-purifying with SLBP (Figure 3.14b). These results provide evidence that SLBP is found in the same protein complex as WDR23 in vivo.

Next, we explored the possibility of SLBP as a substrate of CRL4WDR23. To test this, the active CRL4WDR23 ligase, which includes CUL4A, FLAG-tagged DDB1, WDR23 and RBX1, was purified from Sf9 cells (Figure 3.15a). We also separately purified the potential substrate SLBP (Figure 3.15b). The ligase and SLBP were then used in in vitro ubiquitylation reactions. SLBP, E1, E2 (UbcH5) and ubiquitin were incubated together with and without CRL4WDR23. We found that SLBP was efficiently ubiquitylated by CRL4WDR23 in vitro, and the ligase seems to catalyse attachment of one to several ubiquitin molecules to SLBP (Figure 3.15c).

WDR23-dependent in vivo ubiquitylation of SLBP was also investigated by ubiquitin remnant immunoaffinity profiling of WDR23-dependent lysine ubiquitylation. Briefly, cells depleted of WDR23 and treated with proteasome inhibitor were resuspended in denaturing urea buffer and chromatin sheared by sonication. After typsin digestion of the extract, di-gly peptides were enriched using a specific antibody that recognises di-gly branched peptides. The eluted peptides were then analysed by quantitative proteomics. Using this approach, SLBP lysine 156 ubiquitylation was identified and upon WDR23 depletion the amount of this modified peptide
was reduced (Figure 3.15d). The MS1 chromatographic intensities for an unmodified Actin peptide as well as K63- and K48-linked ubiquitin peptides are shown to demonstrate that the overall peptide pool and ubiquitin enrichment did not fluctuate with the same pattern as that seen for SLBP K156 peptide (Figure 3.15e,f,g).

SLBP Lysine 156 is situated in the central portion of the protein in an α-helical region. Based on the crystal structure of the SLBP RNA binding-domain with histone mRNA stem-loop and the human 3’hExo exonuclease, the side-chain of K156 is solvent exposed and seems to be well-positioned for access by the ubiquitin ligase (Figure 3.15h) (Tan et al., 2013).

The expression of SLBP is only induced in late G1 and during S phase. Upon completion of DNA replication the protein is degraded. A phosphodegaron is responsible for triggering SLBP degradation via polyubiquitylation by the SCF (Zheng et al., 2003). Thus, we hypothesise that CRL4WDR23 ubiquitylation of SLBP does not lead to its degradation. Indeed when the substrate adaptor WDR23 was downregulated, the basal levels of SLBP in cells did not change indicating SLBP ubiquitylation by CRL4WDR23 likely performs a non-proteolytic regulatory function (Figure 3.15i).
Figure 3.11 | SLBP depletion leads to accumulation of uncleaved histone mRNAs.

HeLa cells were treated with SLBP siRNA for 72 h and 3’ uncleaved histone mRNA levels were quantified via qRT-PCR. Data shows mean relative to control across independent experiments with SEM.
Results II: CRL4WDR23 ubiquitylates SLBP for efficient histone mRNA processing

Figure 3.12 | SLBP is mislocalised upon WDR23 depletion.

(a) RNAi treatment was carried out for 72 h and cells fixed for immunofluorescence. Specific antibody against SLBP was used to visualise the subcellular localisation of the protein. Nuclei were visualised using DAPI staining of DNA. (b) The nuclear versus cytoplasmic intensity ratio in cells were analysed in ImageJ. Graph shows mean with SEM. *** p<0.001
Figure 3.13 | WDR23 expression increases in late G1 and S phase.

Cells were synchronised by double-thymidine block and released into S phase. Samples were harvested and extracts made at the indicated time points. Extracts were immunoblotted using cyclin A and GAPDH antibodies as cell cycle and loading controls respectively. Specific WDR23 antibody was used to detect levels of this protein.
Results II: CRL4WDR23 ubiquitylates SLBP for efficient histone mRNA processing

![Figure 3.14](image)

**Figure 3.14** | **CRL4\(^{\text{WDR23}}\) interacts with SLBP in vivo.**

(a) HeLa cells stably expressing doxycycline-inducible strep-strep-HA tagged WDR23 was immunoprecipitated using anti-HA antibodies. Antibodies against WDR23, SLBP, DDB1 and CUL4A were used to assess presence of these proteins in the immunoprecipitates. GAPDH was included as loading control. (b) Specific antibody against SLBP was used to immunoprecipitate endogenous SLBP from total HEK 293 cell extract with unspecific IgG used as a control. The immunoprecipitates were analysed using antibodies against WDR23 and DDB1.
Results II: CRL4WDR23 ubiquitylates SLBP for efficient histone mRNA processing

Figure 3.15 | CRL4WDR23 ubiquitylates SLBP for non-proteolytic regulation.

(a,b) CRL4WDR23 with RBX1 and SLBP were purified from Sf9 cells by FLAG purifications. Contaminants are marked by *. (c) SLBP was incubated with ubiquitin, E1 and E2 (UbCH5) with and without RBX1-CRL4WDR23 at 37°C for indicated amounts of time. Specific antibodies against SLBP, WDR23 and CUL4A were used to detect upshift of band due to ubiquitylation. (d,e,f,g) Ubiquitin remnant immunoaffinity profiling of WDR23-dependent lysine ubiquitylation. Graphs show MS1 chromatographic intensities of peptides from (d) ubiquitylated SLBP K156, (e) Actin (f) ubiquitin K63 and (g) ubiquitin K48. Note: the quantitation of the ubiquitin K48 peptide in (g) is likely inaccurate due to the overwhelming intensity of the K48 peptide in the chromatography. This peptide abundance was much higher than any other signal in the sample causing it to elute as carryover after it’s normal elution time inducing potential inaccuracies in the quantifications.

(h) Crystal structure of SLBP RBD (yellow) with histone mRNA stem-loop (grey) and endonuclease 3’hExo (lilac). Position of K156 is circled in red with side chains of surrounding lysines in cyan. (h) HeLa cells were depleted of WDR23 and basal levels of SLBP were assessed using SLBP antibody. GAPDH was used as loading control.
3.3 Discussion

3.3.1 WDR23 is required for efficient histone mRNA processing

We profiled the downregulation phenotypes of WDR23 and have identified a novel function of CRL4<sup>WDR23</sup> in histone mRNA processing. WDR23 downregulation leads to reduced levels of processed mature histone mRNAs in cells and simultaneous strong accumulation of uncleaved histone mRNAs (Figure 3.4c,d; Figure 3.5). These histone mRNA processing defects are rescued by expression of RNAi-resistant WDR23 (Figure 3.6b,c), confirming the specificity of the defect for WDR23 knockdown. As a consequence of reduced histone transcripts, depletion of the histone pool is also observed at the protein level for all 5 canonical histones upon prolonged depletion of WDR23 (Figure 3.4a).

The reduced ability of cells to produce mature histone mRNAs, and therefore histone proteins, upon WDR23 depletion results in severe and global inhibition on DNA replication as determined by DNA fibre and BrdU incorporation experiments (Figure 3.1d,e,f). Once cells enter S phase it is imperative that replication of the entire genome and re-establishment of chromatin structure is properly completed. In response to reduced histone supply upon WDR23 depletion, replication stress signalling occurs as evidenced by the presence of elevated CHK1 S345 and RPA2 phosphorylation (Figure 3.1g). This slows down S phase and replication fork progression as a short-term response in order to gain time and allow histone supply to recover (Figure 3.1c). Histone supply has previously been reported to regulate replication fork speed and S phase timing (Günesdogan et al., 2014; Mejlvang et al., 2014). Checkpoint activation, particularly CHK1
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phosphorylation, in response to reduced histone supply or deposition to slow down S phase has also been documented (Abe et al., 2011; Groth et al., 2007; Günesdogan et al., 2014; Mejlvang et al., 2014). Thus the secondary cell cycle defects set off by the primary histone supply defect upon WDR23 downregulation are consistent with earlier reports. The ultimate result of insufficient histone supply for DNA replication in WDR23 depletion is that growth and survival of cells are strongly compromised (Figure 3.1a).

3.3.2 CRL4WDR23 ubiquitylates SLBP to maintain normal histone mRNA processing

We showed that WDR23ΔN mutant, which does not form an active E3 ubiquitin ligase with DDB1 and CUL4, behaves in a dominant-negative fashion to mimic the same histone processing defects as WDR23 depletion (Figure 3.9). The resulting decrease in histone supply then induces strong cell cycle and growth defects (Figure 3.9; Figure 3.8). This provides evidence that the function of WDR23 in maintaining efficient histone mRNA processing requires formation of the active CRL4WDR23 E3 ligase for ubiquitylation.

SLBP functions to regulate histone mRNA expression and is necessary for efficient histone mRNA 3' end processing. The knockdown phenotype of SLBP, where uncleaved histone mRNA accumulates mimics that of WDR23 depletion, suggesting a close functional relationship between the two proteins (Figure 3.11). We show that CRL4WDR23 binds SLBP to target it for ubiquitylation in vitro (Figure 3.14; Figure 3.15). Moreover, we investigated ubiquitylation of SLBP in vivo by ubiquitin profiling. This revealed that SLBP K156 is modified by attachment of ubiquitin in a WDR23-dependent manner,
suggesting that this lysine residue is a primary ubiquitylation site involved in regulation of histone mRNA processing (Figure 3.15). Indeed, K156 is exposed in the structure of SLBP bound to histone mRNA stem-loop and thus well-positioned for the addition of a ubiquitin molecule (Figure 3.15). Currently, SLBP K156 lysine mutants are being constructed and confirmation of its functional relevance in vivo will be carried out. Importantly, this lysine residue lies in the RNA binding domain of SLBP (aa128 to 198), which is a highly conserved region of the protein required for both efficient binding and 3’ end cleavage of histone mRNAs. Therefore, modification of this region is likely to have a profound effect on histone mRNA processing.

Ubiquitylation is best known for its function in targeting modified proteins for proteasomal degradation. We found no evidence that CRL4WDR23 targets SLBP for polyubiquitylation and degradation (Figure 3.15i). Moreover, depletion of SLBP mimicked WDR23 depletion phenotypes so rather presence of ubiquitin-modified SLBP and not its degradation plays a role in histone mRNA processing. Thus modification of SLBP by CRL4WDR23 on K156 likely leads to other non-proteolytic regulatory events.

The expression of SLBP is strongly boosted during S phase when replication is active. During this time, it is localised mainly to the nucleus, with low levels of the protein also found in the cytoplasm (Erkmann et al., 2005a). In support of a non-proteolytic mechanism of CRL4WDR23-mediated SLBP ubiquitylation, we also observed that WDR23 depletion led to a predominantly cytoplasmic mislocalisation of the protein, highlighting disruptions to the histone mRNA processing pathway (Figure 3.12).

We conclude that CRL4WDR23 ubiquitylates SLBP and that this modification is required to maintain histone mRNA processing efficiency to supply sufficient histones for normal DNA replication to occur.
3.3.3 Possible mechanism for SLBP ubiquitylation by CRL4WDR23 in histone mRNA processing

Non-proteolytic regulation by ubiquitylation is often utilised to change protein-protein interactions and recruit new interaction partner in a controlled fashion. Catalysis of histone mRNA 3’ end processing requires the step-wise recruitment of large protein and snRNP complexes. It is possible that SLBP ubiquitylation is necessary to bring in a particular factor for the ordered formation of a catalytically competent 3’ end processing complex. Upon adaptor depletion, the formation of the cleavage complex may not proceed as normal, leading to a reduction in histone mRNA processing activity. As 3’ end cleavage of histone mRNAs is not a prerequisite for their export, we hypothesise that in WDR23-depleted cells, SLBP bound to uncleaved mRNAs may be exported and become blocked in translation, resulting in its cytoplasmic localisation (Erkmann et al., 2005b).

We are currently investigating this possible mechanism using quantitative mass spectrometry analysis of proteins co-immunoprecipitating with SLBP under control versus WDR23-depletion conditions. We envision that this approach will allow us to discern whether recruitment of particular processing factors is impaired in the absence of SLBP ubiquitylation.

Ubiquitin binding domains (DBDs) function to recognise ubiquitin-modified proteins. They are divergent in both sequence and structure. Various modes of interaction have been discovered between UBDs and ubiquitin, and around 20 UBD protein families have been described so far and the list of ubiquitin binding proteins is ever increasing (Dikic et al., 2009; Husnjak and Dikic, 2012). Since CRL4WDR23 likely ubiquitylates SLBP for non-
proteolytic regulation, we can assume that a UBP is involved and interaction of this protein with ubiquitylated SLBP is part of the functional mechanism. We are currently exploring the possibility that one of the factors recruited to SLBP-bound histone mRNA stem-loop contains a UBD which would bind the ubiquitylated SLBP.

The binding of a UBP to the ubiquitylated substrate can result in different regulatory consequences. Besides the simple recruitment of the UBP there can also be a change in its activity. The essential role of ubiquitin in intron-containing pre-mRNA splicing has been previously described and represents a relevant example where the binding of the UBP to a ubiquitylated substrate induces a change in its activity (Bellare et al., 2005; 2008).

The removal of an intron is catalysed by the spliceosome, which is composed of five snRNPs (U1, U2, U4, U5 and U6). Catalysis requires the recruitment and rearrangement of the large snRNP complexes which leads to the assembly of the active spliceosome (Staley and Woolford, 2009). Prp8 is an RNA-binding protein central to spliceosome assembly and catalysis of pre-mRNA splicing (Grainger and Beggs, 2005). Ubiquitylation of Prp8 has been found to suppress the activity of RNA helicase Brr2, which unwinds U4 and U6 snRNAs to aid spliceosome assembly (Bellare et al., 2008). With the emergence of more details on 3’ end processing of histone mRNAs, it is now clear that many components are shared between histone mRNA processing and poly(A) mRNA splicing. This includes the surprising finding that the U2 snRNP is recruited to histone mRNA to stimulate 3’ end cleavage (Friend et al., 2007; Marzluff, 2007). In the same study it was found that an RNA helicase hPrp43 is also recruited to the histone mRNA processing complex to stimulate 3’ end formation (Friend et al., 2007). It is thus possible that SLBP ubiquitylation regulates the activity of this helicase in histone mRNA
Results II: CRL4WDR23 ubiquitylates SLBP for efficient histone mRNA processing

processing similar to Prp8 ubiquitylation-mediated regulation of Brr2 helicase in pre-mRNA splicing.

CUL4 is known to be important for many chromatin transactions including genome replication. One of its functions is the CUL4/DDB1-dependent assembly of nucleosomes (Han et al., 2013). Deposition of histone H3-H4 dimers onto nascent DNA from histone chaperone Asf1 requires CUL4-dependent ubiquitylation of acetylated nascent H3 (Han et al., 2013). This modification reduces the affinity of H3-H4 for histone chaperones and is essential for nucleosome assembly during DNA replication. In this study we now find that CUL4 also regulates the production of histones themselves in addition to their deposition as nucleosomes (Han et al., 2013). Together this shows, perhaps not surprisingly, that the availability and deposition of histones are co-regulated by CUL4 where specificity is elegantly dictated by distinct substrate adaptors. It is essential that various stages of histone metabolism does not become uncoupled. Lack of histones is detrimental to DNA replication but an excess of free histone, particularly if not bound to histone chaperones, can be highly toxic. Thus controlling and streamlining both the production and deposition of histones during S phase is crucial and we propose that CUL4 acts as a central regulator in histone metabolism during DNA replication.
3 Results II: CRL4WDR23 ubiquitylates SLBP for efficient histone mRNA processing

Figure 3.16 | Possible CRL4\textsuperscript{WDR23} mechanism in histone mRNA processing.

CRL4\textsuperscript{WDR23} ubiquitylates SLBP and stimulate 3' end cleavage. We hypothesise the ligase may be involved in recruitment of specific histone mRNA processing factors during cleavage complex formation. See discussion text for details.
4 Results III: WDR42a functions in homologous recombination to maintain genome integrity

4.1 Introduction

After DNA replication begins, availability of the sister chromatid as a template opens the possibility of accurate homologous recombination repair of DSBs. Outside of S and G2 phases, HR repair is suppressed. The first step of HR is resection of the DSB ends to expose ssDNA which is bound by RPA (Wold, 1997). RPA is then exchanged for RAD51 which binds to ssDNA to form filaments to invade sister chromatid for synthesis and repair of the DSB (McIlwraith et al., 2000; West, 2003).

From an RNAi live-cell imaging screen, we have identified putative CUL4 adaptor WDR42a as a factor required for normal S phase progression (Piwko et al., 2010). Through in-depth phenotypic analyses of WDR42a downregulation, we show that this adaptor contributes to efficient homologous recombination repair of DNA DSBs to protect the genome from persistent DNA damage.
4.2 Results

4.2.1 WDR42a protects the genome from accumulation of persistent DNA damage

WDR42a is a 67kDa protein and has a structure typical for CUL4 adaptors. It has seven WD40 repeats spanning a large proportion of the protein forming beta-sheets. It has an N-terminal helix-loop-helix domain which confers linker protein DDB1 binding (Figure 4.1a). It was shown to be a putative CUL4 adaptor in previous works (Bennett et al., 2010; Lee and Zhou, 2007), and this is also confirmed here where both DDB1 and CUL4A were found to be stable interactors when SSH-tagged WDR42a is immunoprecipitated (Figure 4.1b). It is also known that this protein contains nuclear localisation and export signals and that it may shuttle between the nucleus and the cytoplasm (Figure 4.1a) (Wu et al., 2012).

During candidate confirmation this adaptor stood out as its downregulation led to a high number of apoptotic cells and resulted in increased appearance of 53BP1 and γH2AX foci. In addition, this protein was previously found to co-purify with DDB1 and ATM in the MitoCheck project (www.mitocheck.org). Thus we found WDR42a to be a very intriguing CUL4 adaptor which had remained uncharacterised.

First the DNA damage and checkpoint activation phenotypes of WDR42a depletion were determined. The cell cycle distribution of DNA damage signalling was analysed in WDR42a-depleted cells by flow cytometry and this revealed γH2AX staining in 25% of cells across the cell cycle (Figure 4.2a). For further characterisation we raised an antibody against the protein using two 15-amino-acid peptides from its N-terminal region. This yielded polyclonal antibodies which strongly detect a band at around 70kDa which is
specifically reduced upon treatment with WDR42a siRNA (Figure 4.2b). When WDR42a was depleted, phosphorylation and activation statuses of multiple DNA damage checkpoint proteins were analysed (Figure 4.2b). Phosphorylation of CHK1 on Ser 345, CHK2 on Thr 68 and ATM on Ser1981 were all increased, suggesting elevated levels of checkpoint activation and appearance of DNA damage (Figure 4.2b). The presence of DSBs was confirmed by pulsed-field gel electrophoresis (Figure 4.2c,d). An increase in levels of cleaved Caspase-3 was also detected upon adaptor knockdown (Figure 4.2b). This is in corroboration with the observation that there is increased apoptosis in cells from the RNAi screen (Figure 2.4). And finally, cell survival of WDR42a-depleted cells were significantly reduced in clonogenic assays (Figure 4.2e,f).

To probe what the primary defect in WDR42a-depleted cells could be, drug sensitivity assays were performed. HeLa cells were treated with siRNA targeting WDR42a and seeded into wells containing increasing concentrations of DNA damage-inducing drugs. Colony formation was determined to assess drug sensitivity. We found that WDR42a depletion rendered cells sensitive to radiomimetic drugs which induce DSBs, such as bleomycin (Figure 4.3a,b). Sensitivity to stalling of replication forks was also tested by treating WDR42a-depleted cells with low nanomolar concentrations of CPT. Here no significant sensitivity was seen (Figure 4.3c,d).

Using the polyclonal antibody against WDR42a, the subcellular localisation of the protein was visualised by immunofluorescence microscopy. This led to the observation that WDR42a is predominantly present in the nucleus and in addition to a pan-nuclear staining, the protein forms nuclear foci. To further investigate the role of WDR42a in DNA damage repair, the response of the protein to induction of different types of DNA
damage was assessed by immunofluorescence visualisation of WDR42a. Complementary to the finding that WDR42a-depleted cells are sensitive to induction of DSBs, we found that treatment of cells with high concentrations of HU and bleomycin, both DSBs-inducing drugs, led to an increase in the number of WDR42a foci in the nucleus (Figure 4.4a,b). However, low concentrations of CPT, which stall replication forks, did not result in significant changes in WDR42a foci (Figure 4.4a,b). Interestingly, the total level of WDR42a did not change upon drug treatment (Figure 4.4a,c). In the same experiment, the DNA damage marker γH2AX was observed to respond to both replication fork stalling and DSBs as expected, with an increase in its global level as well as number of foci formed (Figure 4.4a,d,e).

These results indicate that upon downregulation of WDR42a there is an accumulation of spontaneous DSBs which activates the DNA damage checkpoint and induces apoptosis. The sensitivity of knockdown cells to bleomycin and the observed increase in WDR42a nuclear foci in response to DSBs suggest that the protein plays a role in repair of DSBs.
Figure 4.1 | WDR42a is a putative CUL4 adaptor.

(a) Domain structure of adaptor WDR42a. (b) SSH-tagged WDR42a was stably expressed in HeLa cells from a doxycycline-inducible promoter and immunoprecipitated. Specific DDB1 and CUL4A antibodies were used to probe co-purifying proteins. NES, nuclear export signal. NLS, nuclear localisation signal.
Results III: WDR42a functions in homologous recombination to maintain genome integrity

Figure 4.2 | Downregulation of WDR42a leads to appearance of excessive DNA damage.

(a) RNAi depletion was done for 72 h in HeLa cells. Fixed cells were stained with γH2AX-specific antibody and DNA stained with propidium iodide. Cells were assayed by flow cytometry and analysed in FlowJo. The CPT sample was treated with 1μM CPT for 60 m. (b) Extract from WDR42a-depleted HeLa cells were immunoblotted for DNA damage markers using specific antibodies as indicated. Tublin was included as loading control. (c) After WDR42a depletion chromosomal DNA was separated by pulsed-field gel electrophoresis and the band corresponding to DNA DSBs is labelled. CPT treatment was done using 1μM CPT for 60 m. (d) Intensities of the band corresponding to DSBs was quantified in ImageJ to reflect relative levels of DNA breaks. The majority of chromosomal DNA which remains in the wells was used as loading control for normalisation. (e,f) HeLa cells depleted of WDR42a were seeded into 6-well plates and incubated for 7 days. Colonies were stained and ImageJ was used for assessment of colony formation via quantification of colony intensities.
Results III: WDR42a functions in homologous recombination to maintain genome integrity

Figure 4.3 | WDR42a depletion renders cells sensitive to DSBs.

(a,b,c,d) After 72 h of WDR42a downregulation, HeLa cells were seeded into 6-well plates with increasing concentrations of drugs as indicated. Cells were incubated for 7 days and colonies stained. Image analysis was done in ImageJ by quantifying colony intensities. Graphs show mean with SEM.
Results III: WDR42a functions in homologous recombination to maintain genome integrity

Figure 4.4 | WDR42a functions in DNA damage response to DSBs.

(a,b,c,d,e) HeLa cells were treated with various DNA damage-inducing drugs including 50nM CPT for 3 h to induce stalled replication forks, and 2mM HU overnight or 1μg/ml Bleomycin for 3 h both of which induce DSBs. Cells were fixed for immunofluorescence where WDR42a and γH2AX foci were visualised using specific antibodies. The number (b,d) and total intensity (c,e) of both WDR42a and γH2AX foci were quantified in CellProfiler. Data in graphs are represented as box plots showing mean with 25th and 75th percentiles. The whiskers show 10th and 90th percentiles. * p<0.05, ** p<0.01, *** p<0.001. Immunofluorescence staining was performed by Wojciech Piwko.
4.2.2 WDR42a contributes to homologous recombination repair efficiency

There are two major pathways which repair and clear away DNA DSBs, namely NHEJ and HR. The former is the error-prone repair mechanism and is active prior to DNA replication. HR is active during S and G2 phases as it requires the presence of sister chromatid for error-free repair. As WDR42a downregulation led to accumulation of unrepaired DSBs, we next investigated whether WDR42a functions in the HR pathway.

A U2OS HR reporter cell line was used to assess the repair efficiency of WDR42a-depleted cells. Briefly, the system is based on introducing DSBs via the I-SceI endonuclease specifically in mutated GFP inserts. Efficiency of HR repair is then assayed by flow cytometry detection of GFP expression enabled by HR repair of the cleaved mutant GFP sequences. I-SceI in the reporter cell line was expressed from a doxycycline-inducible promoter. We found that knockdown of the adaptor led to around a 50% reduction in HR repair (Figure 4.5). CtIP, which is required for the initial resection of DSBs in HR repair, led to around 75% reduction in HR upon its depleted.

RAD51 filament-mediated strand invasion is a key step in HR repair of DSBs. Preliminary experiments have revealed that loading of RAD51 may be defective upon WDR42a downregulation. Here, cells depleted of WDR42a were treated with low concentrations CPT, which causes stalling of replication forks that require RAD51 loading and HR repair. RAD51 foci were quantified in cells exhibiting strong γH2AX staining, which were positive for replication stress. It was found that depletion of WDR42a led to a reduction in both the number and size of RAD51 foci relative to control cells upon
replication fork stalling (Figure 4.6). This result provides an indication that during HR repair of stalled replication forks WDR42a is involved in RAD51 loading.

Together, these results show WDR42a may contributes to formation of RAD51 foci during homologous recombination. Upon the knockdown of this CUL4 adaptor, HR repair efficiency was significantly reduced.

**Figure 4.5 | WDR42a contributes to efficient homologous recombination.**

(a) WDR42a was depleted in U2OS HR reporter cells for 48 h and expression of SceI nuclease was induced by doxycycline addition for the last 24 h of downregulation. Cells were trypsinised and assayed by flow cytometry. (b) HR efficiency was calculated by normalisation to background GFP signal in cells without doxycycline (-Dox) (b). Percentage HR efficiency is expressed relative to control.
4.2.3 WDR42a interaction with ATM

ATM is an important regulator of the DNA damage checkpoint and one of its functions is to facilitate end resection in HR repair. It was reported in the Mitocheck project that ATM is an interactor of WDR42a. Hence, we decided to investigate this possibility. First, a stable HeLa cell line expressing doxycycline-inducible SSH-tagged WDR42a was created. WDR42a was immunoprecipitated in the presence and absence of DNA damage induction and a small amount of ATM was detected co-immunoprecipitated (Figure 4.7a). The amount of ATM co-purifying with WDR42a was increased upon induction of DSBs with etoposide treatment, which along with bleomycin are
both radiomimetic drug (Figure 4.7a). We then stably expressed SSH-tagged WDR42aΔN mutant in HeLa cells. This mutant has an N-terminal deletion of the helix-loop-helix domain and has lost DDB1-binding activity (Figure 4.7b). Immunoprecipitation of the ΔN mutant under DNA damage conditions also yielded co-purification of ATM and specifically active ATM phosphorylated on Ser 1981 (Figure 4.7b). Interestingly, overexpression of WDR42a in cells led to decreased basal levels of phospho-ATM without affecting the total ATM levels, alluding to the possibility that WDR42a may negatively regulate levels of active ATM in cells (Figure 4.8).

![Figure 4.7](image_url) | WDR42a interacts with ATM.

(a) WDR42a was immunoprecipitated from HeLa cells stably expressing SSH-tagged WDR42a using anti-HA antibody with and without etoposide. Cells were treated with 20μM etoposide for 1 h prior to harvesting. The presence of copurifying ATM was assessed by immunoblotting using specific antibody (b) WDR42aΔN mutant protein was stably expressed in HeLa cells and immunoprecipitated after DNA damage induction using 1μM CPT for 1 h. DDB1 CUL4A, ATM and pATM S1981 antibodies were used to detect co-immunoprecipitating proteins.
Results III: WDR42a functions in homologous recombination to maintain genome integrity

4.3 Discussion

From manual analysis of images from the RNAi screen and further downregulation phenotype characterisation, it emerged that WDR42a functions in the DNA damage response. Its downregulation led to accumulation of spontaneous DSBs (Figure 4.2c,d) which activate the DNA damage checkpoint (Figure 4.2a,b). The persistent DSBs then trigger apoptosis due to reduced ability of cells to repair and clear away the damage (Figure 4.2b,e,f; Figure 2.4).
We showed that WDR42a-downregulated cells were sensitive to DSB-inducing drug bleomycin (Figure 4.3a,b) but not to replication fork stalling induced by low concentrations of CPT (Figure 4.3c,d) suggesting the repair capability for DSBs specifically are impaired. Furthermore, in corroboration with results from the drug sensitivity assay, cells treated with DSB-inducing drugs including bleomycin and HU led to an increase in the number WDR42a foci in the nucleus (Figure 4.4a,b). By utilising the homologous recombination reporter assay, we found that WDR42a knockdown indeed led to reduced rates of homologous recombination repair, the error-free DSB repair pathway (Figure 4.5). Loading of RAD51 is an important step preceding strand invasion in HR. Our preliminary data shows that loading of RAD51 during replication fork stalling is reduced upon WDR42a depletion (Supplementary figure 4.7). This leads us to hypothesise that perhaps RAD51 filament formation during HR repair of DSBs may also be defective upon adaptor downregulation. In order to confirm this, assessment of WDR42 dependent RAD51 foci formation will be investigated under conditions of S phase specific DSB induction.

In agreement with previous report, ATM was detected co-purified with WDR42a-SSH and this binding appeared to be increased upon DSB induction (Figure 4.6a) (www.mitocheck.org). We then immunoprecipitated the WD42aΔN-SSH mutant, which does not bind DDB1, under conditions of DNA damage and found it to also interact stably with active ATM (Figure 4.6b). These results raise the alluring possibility of ATM or perhaps phosphorylated ATM as a potential ubiquitylation substrate of CRL4WDR42a.

After detailed phenotypic analyses, we can show that WDR42a functions to maintain effective HR repair of DNA DSBs and this may be done by ensuring the formation of sufficient RAD51 filaments to catalyse strand invasion. When this fails upon WDR42a depletion, spontaneous DSBs
accumulate to an excess and trigger apoptosis. To improve our understanding of the functional role played by WDR42a in HR, the early steps of this pathway will be dissected in WDR42a-depleted cells. RPA foci formation in adaptor-depleted cells with DNA damage will be assessed by immunofluorescence to determine if RPA binding to ssDNA is perturbed. A decrease in RPA staining would point to deficiencies at the stage of end resection. Increased RPA foci formation may mean that WDR42a functions to boost RAD51 filament formation, which is a heavily regulated step of HR where both positive and negative mediators are involved in promoting and dissociating RAD51 binding to ssDNA.

A prevalent theme in HR repair is the regulation of repair pathway choice between NHEJ and HR, and this is primarily done at the step of DSB resection. 53BP1 has been suggested to promote NHEJ by increasing the mobility of DSB ends to aid ligation (Bunting et al., 2010). Two proteins Rif1 and PTIP function in association with 53BP1 to block resection (Daley and Sung, 2014). BRCA1, which facilitates CtIP recruitment and DSB resection, may antagonise 53BP1 to boost resection and HR (Bunting et al., 2010; Huen et al., 2009). So one possibility is that CRL4$^{WDR42a}$ may act to boost DSB resection to push repair down the HR pathway, perhaps in the regulation of pathway choice. This hypothesis can begin to be addressed by probing the level of activity of NHEJ using reporter cell lines upon WDR42a depletion and overexpression.

Since the interaction between the adaptor and ATM is increased upon DSB induction and overexpression of WDR42a led to a decrease in the pool of active ATM, one can speculate that CRL4$^{WDR42a}$ may target the protein as a substrate. The first step of HR is resection of DSB ends and two proteins involved in initial and extensive resection, CtIP and EXO1 respectively, are
both phosphorylated and activated by ATM (Figure 1.4) (Bolderson et al., 2010; You and Bailis, 2010).

To better understand the functional mechanism of CRL4\textsuperscript{WDR42} in HR, efforts are being made to search for the ubiquitylation substrate of the ligase. Ubiquitin remnant profiling of WDR42a-dependent lysine ubiquitylation will be carried out in order to identify potential ubiquitylation targets of CRL4\textsuperscript{WDR42a} and to determine whether this includes ATM. As WDR42a functions in clearance of DSBs it is likely that the substrate of CRL4\textsuperscript{WDR42a} will only be ubiquitylated in response to damage induction. Additionally, the interaction between ATM and WDR42a appears to be increased upon DSB induction. Thus, ubiquitin profiling for WDR42a will be carried out under normal as well as DNA damage conditions that induce DSBs. The experimental setup could be treatment of control and WDR42a-downregulated cells with CPT, to induce S phase-specific DSBs, and bleomycin, which mimic the effects of ionising radiation to induce DSBs across the cell cycle. And along with cells without DNA damage induction, WDR42a-dependent changes in ubiquitylated targets can be analysed in relation to the cell cycle phase of induced DSBs.

After detailed phenotypic analysis of WDR42a downregulation, we can conclude that the ligase functions in the HR pathway to boost repair activity. Based on the finding that WDR42a depletion leads to less efficient RAD51 loading during induction of replication fork stalling, we hypothesise that this protein may also be required for RAD51 filament formation for repair of DSBs. These findings demonstrate a previously undescribed function of CUL4 in homologous recombination and exemplify a new way by which CUL4 contributes to genome stability.
5 Final discussion and future perspectives

This doctoral project began by following up on interesting CUL4 adaptors identified from an RNAi screen which are required for S phase progression. Subsequently two adaptors WDR23 and WDR42a were chosen and their functions subjected to detailed characterisation.

Here, functional characterisation of the E3 ubiquitin ligase CRL4\textsuperscript{WDR23} was presented. The ubiquitylation activity of this E3 ligase was found to be required for efficient histone mRNA processing to produce sufficient histone supply to ensure normal DNA replication. The target of its ubiquitylation activity was identified as the stem-loop binding protein, which is important for regulating the entire life cycle of histone production from the stability and 3’ end processing of the mRNAs to ensuring their efficient translation.

The qRT-PCR-based readout for histone mRNA processing, while quantitative, is indirect. In order to unequivocally demonstrate that CRL4\textsuperscript{WDR23}-mediated ubiquitylation of SLBP is required for histone mRNA processing, in vitro mRNA cleavage could be performed using control and WDR23-depleted nuclear extracts.

As a non-proteolytic regulation of SLBP by the ligase is proposed, it will be of keen interest to identify the potential binding partner of modified SLBP.
This will be done either by MS analysis of WDR23-dependent changes in cleavage complex composition or sequence alignment search for potential UBPs in known histone mRNA processing factors. Once potential effector UBPs are identified, follow up investigations could include assessing dependence of UBP binding to SLBP on WDR23 and SLBP K156 mutation, assuming this is the true \textit{in vivo} lysine modified by CRL4$^{\text{WDR23}}$. For the identified UBP, it will also be interesting to analyse deletion mutants of the ubiquitin-binding domain for confirmation.

For WDR42a, the downregulation phenotypes of this CUL4 adaptor was extensively analysed and the adaptor was found to function in homologous recombination repair of DSBs. Adaptor depletion led to reduced HR rates in cells and as a result spontaneous DSBs persist and accumulate triggering cell death.

In order to gain a deeper understanding of the mechanism of WDR42a, two aspects of its function have to be investigated. Firstly, the HR pathway and the role WDR42a plays within it has to be rigorously analysed. A collaboration has been set up with Dr Christoffel Dinant at the Danish Cancer Society Research Centre in Copenhagen to study WDR42a further. Using various cell lines expressing GFP-tagged DNA damage and HR factors, the recruitment of specific damage proteins upon induction of DSBs will be analysed in control and WDR42a-depleted cells. This will provide vital information about the step in HR which is dependent on WDR42a. As NHEJ and HR pathway choice is one possibly for the function of CRL4$^{\text{WDR42a}}$ the share of activities of these two pathways and its dependence on WDR42a will also be probed using report cell lines.

Secondly, substrate identification will be key. Both directed, with the hypothesis that ATM is a substrate, and unbiased, where ubiquitin profiling
is utilised, approaches will be explored. In the directed approach, the \textit{in vitro} binding and ubiquitylation of ATM by the E3 ligase will be assessed. Ubiquitin profiling of WDR42a is planned under normal and DNA damage conditions. Additionally, interactors of WDR42a will also be analysed by affinity purification followed by mass spectrometry under the same DNA damage conditions as ubiquitin profiling. After potential substrates and modified sites are identified their lysine mutants will be phenotypically analysed.

The importance of CUL4 in replication and DNA damage repair is undisputed. The ability of CUL4, or cullins in general, to regulate such a broad spectrum of important processes is down to its ability to associate with an array of substrate adaptors in a modular fashion. This mode of function is a way of creating flexibility and specificity of control in cellular processes to enable finely-tuned, centralised and efficient regulation in response to signals and stimuli. The activity, assembly and disassembly of CRLs is highly regulated and CRL activation cycle regulation is an active and fascinating area of research. Some parts of the model for the activation cycle of CRLs is still in need of \textit{in vivo} validation.

Studies have shown that CAND1 mediates SR module exchange and assembly of new CUL1-based complexes (Pierce et al., 2013). It will be important to validate this mechanism for other cullin complexes, particularly in the case of CRL4 where the adaptor DDB1 is structurally distinct from other cullin adaptors (Lydeard et al., 2013). By extension, it will also be of interest to investigate whether and how the process of CRL assembly \textit{in vivo} is regulated, especially in response to signalling and changes in the cellular environment. One other mystery surrounding this protein is that different cullins are found associated to CAND1 to different extents \textit{in vivo}, pointing to varying levels of control by CAND1 (Bennett et al., 2010).
Another point to address concerns the observation that the binding of neddylated CRLs to the CSN and their subsequent deneddylation may be two separate events (Bennett et al., 2010). This finding raises the possibility that signalling or additional regulators of the CSN deneddylation activity may exist.

With regards to CRL4s, the diversity in binding mechanisms employed by the substrate adaptors for DDB1 association provides intriguing challenges to understanding their distinct functionality among all the cullin-based ligases. One reason for the evolution of multi-surface protein-protein interactions between the three DDB1 β propellers domains and the DCAFs could be to allow for additional regulation of CRL4s. One DDB1-interacting protein, Dda1, which interacts with multiple CRL4s could be a candidate and act as a regulator to modulate adaptor and DDB1 binding (Olma et al., 2009). Interestingly, Dda1 binds to a conserved cluster of residues on the surface of the BPA propeller which is outside the normal BPA-BPC binding pocket employed by other interactor and adaptors for DDB1 interaction (Jin et al., 2006). It will be exciting to see if future investigations do unearth such a regulator of CRL4. Another interesting area of CUL4 research is the question of the respective functions of CUL4A and CUL4B. While the two do share some functionally redundancy, the reason why there exists two homologues of CUL4 but not other cullins is unclear. The function of the additional extreme N-terminal domain of CUL4B requires elucidation.

In the context of individual cullins, combinatorial binding of adaptors via the same cullin scaffold is an elegant way of integrating regulation of processes which may affect each other or are co-dependent on one another. In the case of CUL4, the same E3 ligase backbone functions in DNA replication and DNA damage responses, two processes which are both vital to the cell’s survival and very closely interconnected. In addition to studying
regulation of the CRL activation cycle, the functional analysis and substrate identification of cullin-based ligases are critical. Out of the hundreds of unique CRLs, target substrates of the majority remain unidentified. This project has led to the characterisation of two CUL4 adaptors with previously unknown functions. It has identified new functions of CUL4 in histone production and homologous recombination repair of DSBs mediated by the adaptors WDR23 and WDR42a.
6 Materials and methods

6.1 Mammalian cell culture

HeLa, U2OS and HEK 293 cells were grown in NUNC cell culture dishes in Dulbecco's modified medium (DMEM) from Invitrogen supplemented with 10% FBS and 1% Penicillin-Streptomycin-Glutamine 100x (PSG, Life Technologies). RNAi experiments were performed with Lipofectamine RNAiMAX (Invitrogen) with 30nN siRNA for 48 or 72 hours and specific antibodies were used to assess downregulation efficiencies. Stable cell lines were generated with FRT-TetR cells using the Flp-In system (Invitrogen) as described previously (Glatter et al, 2009). Cell lines once generated were maintained in 200μg/ml Hygromycin and 10μg/ml Blastici. Transient overexpression was carried out using Lipofectamine 2000 (Invitrogen) following standard manufacturer's protocol.

For clonogenic assays, HeLa or HEK293 cells were subjected to RNAi for 48 hours before being split into six-well dishes (700 or 1000 cells/well). For drug sensitivity testing, various drugs at decreasing concentrations were added to cells. After this, cells were incubated for 7 to 10 days. At the end of the incubation period, the DMEM was removed and wells washed 3 time with PBS and stained with 0.25% crystal violet in 80% methanol. Colonies were then counted.
For cell synchronisation by double thymidine block and release into S phase, cells were treated with siRNA for 7 hours then 3mM thymidine was added to the plates and cells incubated for 16 hours. Cells were washed three times with warm DMEM (37°C). This release was done for 10 hours followed by a second 16-hour period of 3mM thymidine treatment. Release was again done via 3 consecutive washes with warm DMEM before harvesting of cells at different time points. Synchronisation of cells in mitosis was done with thymidine treatment followed by nocodazole. Cells were subjected to RNAi for 7 hours before being incubated with 3mM thymidine for 24 hours. Thymidine was washed out 3 times using warm DMEM and cells released in incubation for 5 hours followed by addition of 75ng/ml of nocodazole and incubation for 10 hours. Three washes with DMEM was then done for final release and cells were harvest at various time points.

The homologous recombination reporter assay was carried out using U2OS DR-GFP/tetR/LacO-I-SceI cells. After siRNA treatment for 24 hours, doxycycline was added at 1μg/ml to induce expression of the Scel nuclease and cells incubated for a further 48 hours. RNAi treatment conditions were done in duplicates where only one is induced by doxycycline and the un-induced acted as a control for background GFP signal. For analysis, cells were trypsinised and resuspended in PBS and GFP signal quantified using the FACSCalibur flow cytometer (BD Biosciences) and FlowJo software.

6.2 Antibodies

Specific antibody against WDR42a was generated with Eurogentec using their standard peptide antibody generation programme. Two peptides of 15 amino acids in length corresponding to the N-terminus of WDR42a were
inject into two rabbits and the resulting batches of sera were used for peptide antibody purification using WDR42a peptide linked to Amino-Link Plus Coupling Resin (Thermo Scientific). Elution was done with low pH (100mM Glycine pH 2.5).
### 6.2.1 List of antibodies used

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### 6.4 Immunoblotting and immunoprecipitation

Cells were harvested by scraping and pelleted at 500g for 2 minutes at 4°C. The cell pellet was washed once with ice cold PBS and re-pelleted. Cells were then lysed in lysis buffer (20mM Tris pH 7.5, 150mM NaCl, 5mM MgCl₂, 0.1% Triton x100, 10% Glycerol, 20mM beta-glycerophosphate, 0.5mM DTT, 1mM NaF and Roche protease inhibitor cocktail). Lysate was rotated with addition of Universal Nuclease (Pierce, 2μl per each 15cm dish) for 30 minutes at 4°C and sheared using a 26G needle at time 0 and 15 minutes with 5 up-and-down motions. Cell lysate was then cleared by centrifugation at 10,000g at 4°C for 10 minutes. Protein concentration was determined and equalised via the Braford assay. Samples were boiled for 10 minutes at 95°C in 1x LDS (Novagen) and 10mM DTT. Proteins were resolved by SDS-PAGE and transferred to PVDF Immobilin-P membrane (Millipore). Blocking and antibody incubations were carried out in 5% milk (Migros) in PBS-T (0.1% Tween-20).

For immunoprecipitation of strep-strep-HA-tagged proteins. Stable protein bait expression was induced by addition of 1μg/ml of Doxycycline for 12 to 14 hours. Cell pellets were resuspended in IP buffer (10mM Tris pH 7,
100mM Kc, 20mM MgCl₂, 0.5% NP40, 300mM Sucrose, 20mM beta-glycerophosphate, 0.2mM NaF, 0.2mM PMSF and Roche Complete Protease Inhibitor Cocktail tablet). Shearing of chromatin material by needle and lysate clearing were done as in preparation of cell extracts. Anti-HA antibody-coupled beads (Sigma; 12.5 ml beads per 15 cm dish) were pre-equilibrated with IP buffer and lysates incubated with beads for 1.5 hours. Beads were washed with IP buffer four times and eluted with LDS buffer without DTT by boiling at 95°C for 5 minutes. Then 20mM final concentration of DTT was added before analysis by SDS-PAGE and immunoblotting.

For endogenous immunoprecipitation using specific antibodies, cells were harvested and resuspended in EndoIP buffer (50mM HEPES pH7.9, 125mM Potassium acetate, 2mM Magnesium acetate, 7.5% Glycerol, 0.5% NP-40). For each 15cm plate of cells, 5μg of specific-antibody or IgG as negative control was added to the lysate and incubated at 4°C for 1 hour. Pre-equilibrated Protein-A beads (Bio-Rad) were added to the lysate and incubated at 4°C for 1 hour. Immunoprecipitated proteins were eluted with LDS without DTT by boiling at 95°C for 5 minutes and then DTT at 20mM was added after. Analysis was done by SDS-PAGE and immunoblotting.

When crosslinking was performed for immunoprecipitation experiments, DTSSP was used. After cell lysis 0.25mM DTSSP was added and incubated at 4°C for 2 h in the corresponding IP buffer with only 0.2mM PMSF as the sole protease inhibitor. DTSSP was then quenched using 1M Tris pH7 for 15 minutes before proceeding to cell lysate clearance by centrifugation.
6.5 Immunofluorescence

Cultured HeLa or U2OS cells were grown on coverslips before being fixed, permeabilised and stained with antibodies. Secondary antibodies used were labelled with Alexa fluor 488 or 568 (Invitrogen) and 1μg/ml DAPI was added for detection of nuclei. Coverslips were mounted on glass slides using Immu-Mount (Thermo). Images acquisition was done using Leica DM6000B epifluorescence microscope or Nikon TI eclipse inverted microscope.

For 53BP1 and γH2AX stainings, cells were fixed with methanol for 20min at -20°C and permeabilised with acetone for 2 minutes at -20°C. Coverslips were washed with PBS containing 0.01% Triton X-100 (PBS-0.01%T) three times then blocked and incubated with antibodies in PBS-0.01%T 5% FCS.

For SLBP staining, cells were fixed with 4% paraformaldehyde (PFA) in PBS for 10 minutes at room temperature and permeabilised using 0.2% Triton-X100 in PBS for 3 minutes. Blocking and antibody incubations were done in 2% BAS in PBS-0.01%T.

For RAD51 staining, cells were subjected to pre-extraction using 0.2% Triton in PBS for 3 mins. Fixation was done using 4% PFA.

6.6 DNA fibres

This DNA fibres protocol was kindly shared with us by Kristijan Ramadan (Oxford institute for radiation oncology). Cells were grown and treated with siRNAs in duplicates in six-well plates for 72 hours. One set of samples were pulsed with 30μM CldU for 30 minutes at 37°C, washed and then pulsed with
200μM IdU for 30 minutes at 37°C. All cells were harvested by trypsinisation and resuspended in cold PBS and cell number counted. For each siRNA condition, the labelled and unlabelled cells were mixed 1:5 by cell number. 7.5μl of freshly made lysis buffer (200mM Tris-HCl pH 7.4, 50mM EDTA, 0.5% dust-free SDS) was mixed with 2.5μl of cell suspension on a glass slide. The slide was tilted at 45° for the droplet to roll down the slide and spread the DNA from lysed cells. Slides were air dried and fixed with methanol/acetic acid 3:1 overnight at 4°C. Prior to immunostaining slides were rehydrate in PBS, denatured in 2.5M HCl for one hour and washed with PBS three times. Slides were blocked (40 minutes), stained with primary (2.5 hours) and secondary (1 hour) antibodies in 2% BSA 0.1% Tween 20 in PBS. Primary antibodies used were mouse anti-BrdU/IdU (1:100, Becton Dickson), Rat anti-BrdU/CldU (1:500, Abcam). Secondary antibodies used were anti-mouse Alexa 488 (1:300) and Anti-rat Cy3 (1:300). Slides were washed with PBST (PBS, 0.2% Tween 20) after both primary and secondary antibody incubation 3 times. After air drying slides completely, coverslips were mounted with 20μl of antifade Gold (Invitrogen) per slide then edges were sealed with clear nail polish (Coop).

6.7 Flow cytometry and BrdU incorporation

For cell cycle profiling, cells were harvested by trypsinisation, washed with PBS and resuspended in 300μl cold PBS. 1ml of 100% ethanol at -20°C was added to the cell suspension, vortexed and incubated at -20°C for 2 hours or overnight. Fixed cells were pelleted by centrifugation at 1000g and washed once with PBS and pelleted again. DNA content was then stained with 50μg/ml propidium iodide solution for 30 minutes in the dark for 30
minutes. Samples were analysed with the FACSCalibur flow cytometer (BD Biosciences) using FlowJo software.

For γH2AX staining, cells were fixed and pelleted as above then permeabilised with 5% FCS, 0.25% Triton-X100 in PBS. Then cells were centrifuged and resuspended in 100μl of PBST-FCS (5% FCS, 0.1% Triton-X100 in PBS) containing primary mouse anti-γH2AX. After incubation for 1 hour at room temperature, cells were washed three times with PBST-FCS and staining with secondary anti-mouse AlexaF488 for 1 hour in the dark at room temperature. The DNA content was stained as above.

For BrdU incorporation, cells were pulsed for 30 minutes at 37°C with 30μM BrdU before being harvested, fixed and pelleted as above. Cells were then incubated in 1ml of 2M HCl 0.5% Triton-X100 for 30 minutes at room temperature and the acid neutralised with 2ml 0.1M BORAX. After centrifuging cells, the pellet was washed with 1% BSA in PBS and resuspended in 50μl of 1% BSA, 0.5% Tween-20 in PBS (PBST-BSA) containing anti-BrdU FITC-conjugated antibody for incubation at room temperature for 1 hour in the dark. After washing with PBST-BSA, DNA was counterstained as before.

6.8 qRT-PCR

Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen). Reverse transcription was carried out using random primers and SuperScript II Reverse Transcriptase (Invitrogen) according to manufacturer’s protocol. qRT-PCR was carried out using the LightCycler 480 (Roche Life Science) with the LightCycler 480 SYBR Green I Master (Roche)
Materials and methods

hot start reaction mix. Experiments were done in technical triplicates and normalised to both GAPDH and beta-Actin. A list of primers used are listed below.

6.8.1 List of qRT-PCR primers used

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6.9 Insect cell culture for protein purification

Sf9 cells were grown and maintained in Sf-900 III SFM medium (Invitrogen) with 1% PSG in suspension. For protein expression cells were seeded at 2 million per ml and infected with baculovirus and incubated for 72 hours. Cells were harvested by centrifugation at 4°C, 400g for 3 minutes.

A single step FLAG purification was done for protein purification. Cell pellets were lysed in lysis buffer (50mM Tris-HCl pH7.5, 300mM NaCl, 1mM EDTA, 5mM DTT, 1% Triton-X100 and Roche Complete Protease Inhibitor Cocktail tablet) and homogenised using a 15ml Douce homogeniser with 15 strokes. Lysate was cleared of cell debris by centrifugation at 10,000g, 4°C for 30 minutes. Lysate was added to anti-FLAG beads (Sigma) pre-equilibrated with lysis buffer and incubated at 4°C for 1 hour rotating before washing with lysis buffer and wash buffer (50mM Tris-HCl pH7.5, 150mM NaCl, 1mM EDTA). Bound proteins were eluted with 2mg/ml triple-FLAG peptide in wash buffer twice at room temperature, rotating for 20 minutes. After elution, beads were pelleted by centrifugation at 300g, room temperature for 2 minutes and the supernatant collected.

6.10 In vitro ubiquitylation

500nM SLBP, 100nM UbE1, 400nM UbcH5m 15μM ubiquitin and 40nM CUL4 complex (DDB1, CUL4, WDR23 and RBX1) were incubated in the reaction buffer (50mM Tris pH 7.6, 3mM ATP, 0.5mM DTT, 10mM MgCl2 and 1mg/ml BSA) with a total volume of 50μl at 37°C. At each specified time point, 10μl of the reaction mixture was taken out for analysis by SDS-PAGE and immunoblotting.
6.11 Pulsed-field gel electrophoresis

This protocol was kindly shared by Claus Azzalin (ETH). 1% Low Melting Temperature (LMT) Agarose (FMC, NuSieve GTG Agarose) in PBS was incubated at 50°C in the water bath for 15 minutes. Cells of interest were trypsinised, washed and resuspended in cold PBS. Cell suspension was equilibrated at 50°C for a few seconds and immediately mixed with an equal volume of 1% LMT agarose. The mixture was pipetted into the Plug Mold (Bio-Rad) and incubated at 4°C for 30 minutes. Solidified plugs were ejected into lysis buffer (10mM EDTA, 0.2% Sodium Deoxycholate, 1% Sodium Lauryl Sarcosine and 1mg/ml Proteinase K added freshly) and incubated for 48-72 hours at 37°C. Plugs were washed in wash buffer (20mM Tris pH 8 and 50mM EDTA pH 8) three times for 30 minutes at room temperature rotator. Plugs were inserted into wells in 0.9% Pulse Field Certified Agarose (Bio-Rad) and the pulse field electrophoretic run was performed. The gel was stained with 0.3μg/ml EtBr in TBE for one hour and bands were visualised via UV illumination.

6.12 Ubiquitin remnant immunoaffinity profiling

Ubiquitin profiling protocol from Christopher Barnes.

6.12.1 Harvesting HeLa cells

10 confluent 15 cm plates containing asynchronous HeLa cells corresponding to the two conditions were scraped in their media and 4°C for
3 minutes at 300 rcf (g). Pellets were washed briefly with ice-cold PBS with quick vortexing to dislodge the pellet and again centrifuged at 4°C for 3 minutes at 300 rcf (g). PBS was poured out and the pellets were snap frozen in liquid nitrogen and stored at 80°C prior to lysis and digestion.

6.12.2 Lysis, digestion, peptide desalting, and lyophilisation

Pellets were resuspended in Urea lysis buffer (ULB) containing 9M urea, 50 mM Ammonium Bicarbonate, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, and 1 mM β-glycerophosphate such that the final concentration would be less than 5 mg/mL (each confluent 15 cm HeLa plate yields roughly 1.5-2 mg of protein). Pellets were thawed in hand and then vortexed for 30 seconds until the pellet is thoroughly thawed and suspended. Tubes were allowed to sit on top of ice for 10 minutes before sonication. Sonication was performed with 5 rounds of 1.5 minutes on a Branson 250 tip sonicator with a power output of 15 and a duty cycle of 70%. Between sonication cycles, samples were cooled for at least 2 minutes submerged in ice to reduce heat induced by the process. It is important to take monitor that the sample viscosity has been significantly reduced after sonication, which would suggest that shearing of DNA has occurred at a significant level. Lysed pellets were then centrifuged at 20,000 rcf (g) at 15 °C for 15 min. Supernatants were transferred to a new tube and the remaining cell debris pellet discarded. 100 μL of lysate was saved for BCA analysis to determine protein concentration. Tris(2-carboxyethyl)phosphine (TCEP) was added to the lysate at a final concentration of 10 mM and allowed to stand at room temperature for 30 min. It’s important to ensure that the pH is 7.5 after addition of TCEP. During the TCEP step, BCA was performed to determine protein concentration using the standard manufacturer’s protocol.
Iodoacetamide was added from freshly made stocks (in 100 mM ammonium bicarbonate buffer) to a final concentration of 20 mM with the pH maintained at 7.5 while protecting from light as iodoacetamide is light sensitive. Then, samples were allowed to stand for 30 minutes at room temperature (in the dark). Lysates were diluted to 4M urea with 100 mM ammonium bicarbonate buffer (pH 8) and LysC was added at 1:100 dilution based on protein amount and placed in a shaking incubator at 37°C for 4 hours. Samples were then diluted to a final concentration of 1M urea with 100 mM ammonium bicarbonate and sequencing grade trypsin was added at 1:100 for overnight digestion at 37°C. In the morning, the trypsin reaction was stopped by adding formic acid to a final concentration 1% (pH <3) and samples were placed on ice for 15 minutes to allow precipitate to form. Lysates were centrifuged at 2000 rcf (g) for 15 minutes at room temperature. The cleared protein solution was then transferred to a new tube for peptide desalting. Desalting was performed using c18 columns with a 20 mg capacity connected to a 50 mL syringe. Columns were washed with 5 mL MeOH (1x), 5 mL 50% formic acid (FA) : dH2O with 0.1% FA (1x), and dH2O with 0.1% FA (1 mL, 3 mL, then 6 mL) prior to loading of digested peptides. Peptides were loaded into the back of the syringe and allowed to flow through by gravity with gentle pressure from the syringe plunger as needed. Bound peptides were washed sequentially with 1 mL, 5 mL, and 6 mL of dH2O with 0.1% FA followed by 2 mL of 2% FA : dH2O with 0.1% FA (1x). Finally, new 50 mL tubes were placed below the columns and peptides were eluted with 3 x 3 mL 50% FA : dH2O with 0.1% FA. Samples were stored at 80°C overnight and then transferred to a standard lyophilizer (Christ) in liquid nitrogen and lyophilized for at least 48 hours in order to evaporate all of the formic acid.
6.12.3 Antibody-based immunoprecipitation of K-GG modified peptides

The procedure for antibody-based immunoprecipitation of K-GG modified peptides was used according to the Cell Signaling Technology, Inc. standard protocol for the PTMScan® UbiScan® kit. Briefly, 1.4 mL of the provided immunoaffinity purification (IAP) buffer was added to the lyophilized peptides and centrifuged at 2000 rcf (g) at room temperature for 5 minutes to collect all of the material. Using a P-1000 micropipettor, the sample was mechanically pipetted multiple times with care to not form bubbles and then transferred to a 1.7 mL Eppendorf tube. A small amount of sample was spotted on pH paper to ensure that the FA had been thoroughly removed. The sample should have a pH close to neutral at this step. Samples were cleared by centrifugation at 10,000 rcf (g) at 4 °C for 5 min. During centrifugation, the antibody-bead slurry was washed 4 times with 1 mL of 1X PBS and resuspended in 40 uL PBS for use. The cleared peptide solution was transferred directly on top of the washed antibody-bead slurry careful to not create bubbles, but ensuring mixing of the sample with the beads. Samples were sealed with parafilm and incubated on a rotator at 4°C for 2 hr. After 2 hr incubation, samples were centrifuged 2,000 rcf (g) for 30 seconds and the supernatant (unbound fraction) was transferred to a new labeled Eppendorf tube and stored at -80°C until after analysis. These samples can be enriched for other modifications like phosphorylation if desired. The beads were washed with 1 mL IAP buffer, mixed by inverting 5 times, centrifuged at 2,000 rcf (g) for 30 seconds, and the supernatant was removed and discarded. This IAP wash was repeated for a total of 2 washes followed by three water washes (HPLC-grade) performed with the same procedure. K-GG peptides were eluted by adding 55 µL of 0.15% trifluoroacetic acid (TFA) to the beads and letting stand at room temperature for 10 minutes. The bottom
of the tube was gently tapped to mix the sample every 2-3 minutes during the elution. Tubes were centrifuged at 2,000 rcf (g) for 30 seconds and the supernatant was transferred to a new Eppendorf tube. A second elution using 50 µL of 0.15% TFA was performed with the same procedure and the eluates were combined.

6.12.4 K-GG modified peptide desalting and LC-MS/MS analysis

Eluted K-GG peptides were desalted on StageTips as follows. StageTips were equilibrated with 50 µL 50% acetonitrile (ACN) : dH₂O with 0.1% TFA (1x) followed by 50 µL dH₂O with 0.1% TFA (2x). Samples were loaded in two steps loading 50 µL each time and passing the solution through with a centrifuge at 2,000 rcf (g) for 30 seconds. The bound peptides were washed with 55 µL dH₂O with 0.1% TFA (2x) and eluted with 10 µL 50% acetonitrile (ACN) : dH₂O with 0.1% TFA (2x). Solvents were evaporated in a SpeedVac and peptides were resuspended in 10 µL 2% acetonitrile (ACN) : dH₂O with 0.1% TFA. LC-MS/MS analysis was performed on a Thermo Scientific Q-Exactive in data-dependent analysis mode using a top 20 method and a 1.5 hour gradient of 5-40% acetonitrile. Raw files were converted to mzML using msconvert and searched with Comet as part of the TransProteomicPipeline version 4.7.0. Peptides were filtered for a 1% false discovery rate based on their peptide prophet scores and Skyline version 2.6.0 was used to integrate the area under the MS1 chromatography peaks to generate label-free quantification information. For this, chromatographic traces were aligned and peak areas selected manually for multiple peptides of interest.
References


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Zusammenfassung

Das Protein Cullin4 (CUL4) ist strukturgebend für eine Vielzahl von E3-Ubiquitin-Ligasen und hat eine wichtige regulatorische Funktion während der DANN-Replikation sowie in der DNA-Schadensantwort. Trotz der tragenden Rolle dieser Cullin-4-basierten E3-Ubiquitin-Ligasen (CRL4) sind die molekularen Details ihrer Funktion kaum bekannt.

Um die zelluläre Aufgabe verschiedener CRL4s besser zu verstehen, hat das Labor von Professor Matthias Peter einen Mikroskopie-basierten RNAi-Screen durchgeführt, der darauf abzielte neue S-Phase-spezifische CUL4-Adaptoren zu identifizieren. Im Zuge dessen wurden die zwei neuen CUL4-Adaptoren WDR23 und WDR42a isoliert, die für den Ablauf der S-Phase wichtig sind. Die detaillierte funktionale Charakterisierung dieser Adaptoren ist der Fokus dieser Doktorarbeit.

Für die Assemblierung von Chromatin während der DNA-Replikation benötigt es einen stabilen Pool von nasznten Histonen. Jegliche Änderung der Histonmenge hat fatale Auswirkungen auf Zellzyklus, Genomstabilität und Entwicklung. In dieser Arbeit zeigen wir eine bisher nicht bekannte Funktion der CRL4WDR23-Ligase in der Prozessierung von Histon-mRNA. Unsere Untersuchung hat ergeben, dass die CRL4WDR23-Ligase das „stem-loop“-bindende Protein (SLBP) ubiquitiniert, das seinerseits die Prozessierung und Translation von Histon-mRNA reguliert. Wenn die
Ubiquitinierungs-Aktivität von CRL4\textsuperscript{WDR23} durch die Inhibierung der Komplexformation aufgehoben oder WDR23 herunterreguliert wird, akkumuliert in der Zelle unprozessierte mRNA und die Histonzahl sinkt merklich. Als direkte Konsequenz beobachteten wir eine signifikante Reduktion der Geschwindigkeit von Replikationsgabeln, eine Verzögerung der S-Phase durch die Aktivierung von CHK1 sowie einen generellen Effekt auf das Zellwachstum.

Direkt nachdem die Zelle die DNA-Replikation initiiert hat, werden dabei auftretende Doppelstrangbrüche (DSBs) akkurat mittels homologer Rekombination und naszenter DNA als Vorlage repariert. Die phänotypische Charakterisierung von der Herunterregulierung von WDR42a hat ergeben, dass die Funktion des Proteins entscheidend für die effiziente Reparatur nach dem Prinzip der homologen Rekombination ist. Die Herunterregulierung von WDR42a führt dazu, dass persistierende Doppelstrangbrüche den „DNA-Damage-Checkpoint“ aktivieren und in letzter Konsequenz Apoptose auslösen.

Die Identifizierung und detaillierte Charakterisierung der jeweiligen Funktion der CUL4-Adaptoren WDR23 und WDR42a in der Prozessierung von HistomRNA sowie der homologen Rekombination ermöglichte neue Einsichten in die Funktionsbandbreite der CUL4-basierten E3-Ligasen. Diese Arbeit unterstreicht die Relevanz von CUL4 während der DNA-Replikation und verdeutlicht die funktionale Diversität von CRL4s für einen problemlosen Ablauf der S-Phase. Durch eine Reihe von substratspezifischen Adaptoren kann CUL4 parallel unterschiedlichste Funktionen wahrnehmen und wird so seiner übergeordneten Rolle gerecht, die Chromatin-Integrität zu schützen. Langfristig wird die Charakterisierung von weiteren CUL4-Adaptoren, ähnlich wie in dieser Arbeit aufgezeigt, zu einem besseren
Verständnis der Funktion von CUL4 zur Erhaltung der Genomintegrität führen.
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