The Role of the ASCC Complex in the Alkylation Damage Response

Jennifer Soll
Washington University in St. Louis

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The Role of the ASCC Complex in the Alkylation Damage Response
by
Jennifer Soll

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

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Jennifer Soll

Washington University in St. Louis

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Dedicated to A.J. and Watson. I cannot wait to start our next adventure together.
ABSTRACT OF THE DISSERTATION

The Role of the ASCC Complex in the Alkylation Damage Response

by

Jennifer Soll

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Molecular Genetics and Genomics

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Professor Nima Mosammaparast, Chair

DNA alkylation damage is caused by various agents that are present in the environment as well as cellular metabolism and can be induced by certain chemotherapeutic agents. Thus, the repair of damaged DNA is critical for genomic maintenance. The ALKBH family of proteins plays a central role in the repair of specific alkylated lesions, including 1-methyladenine (1meA) and 3-methylcytosine (3meC). A major outstanding question in the field of alkylation repair is the role of associated protein partners in the function of the human AlkB homologues. Here, I demonstrate that the ALKBH3 associated complex ASCC (comprised of ASCC1, ASCC2, and ASCC3) localizes to distinct nuclear foci specifically upon exposure of cells to alkylation agents. These foci associate with alkylated nucleotides and coincide spatially with elongating RNA polymerase II and splicing components. Recruitment of the repair complex to sites of alkylation damage requires recognition of K63-linked polyubiquitin by the CUE (coupling of ubiquitin conjugation to ER degradation) domain of ASCC2. Mutation of ASCC2 to prevent the binding of K63-linked polyubiquitin results in the impediment of alkylation adduct repair kinetics and increased sensitivity to alkylation agents. This work identifies a previously unrecognized ubiquitin-dependent pathway induced specifically to repair alkylation damage. The ASCC1
subunit is also critical for the regulation of this repair complex. ASCC1 is present at nuclear speckles prior to damage but leaves the foci in response to alkylation. ASCC1 loss significantly increases ASCC3 foci formation during alkylation damage, indicating a negative regulatory function. The research presented herein suggest that ASCC1 coordinates the proper recruitment of the ASCC complex during alkylation, a function that appears to depend on a putative RNA-binding motif near the ASCC1 C-terminus. Together, these findings contribute significantly to the identification of regulators of alkylation damage repair in human cells.
Chapter 1: Introduction


1.1 Alkylation Damage and Repair

The vital importance of genome maintenance in response to damage is underscored by the evolution of multiple highly conserved DNA damage repair mechanisms. These repair mechanisms are often restricted to repairing a specific type or class of damaged DNA. Even within a specific category of DNA damage, the machinery utilized for repair varies due to the context of damage, differential expression or post-translational modification of repair factors, chromatin state, and cell cycle. The complexity and range of damage adducts is particularly evident for DNA alkylation damage. Compounds that transfer alkyl groups to nucleic acids are typically broadly reactive with each capable of producing several distinct DNA lesions (Fu et al., 2012). Sources of damage include the endogenous alkylating agent S-adenosylmethionine as well as exogenous sources, such as tobacco smoke (Hecht, 1999; Rydberg and Lindahl, 1982).

To repair damage, multiple distinct alkylation repair pathways have evolved, giving the cell wide-ranging resources with which to protect its genome. It is important to recognize the clinical relevance of alkylation repair as alkylation-inducing chemotherapeutics are commonly used as part of the cancer treatment arsenal (Drablos et al., 2004; Fu et al., 2012).

The simplest type of DNA alkylation, the transfer of a single methyl group to a DNA base, demonstrates the diverse nature of damage lesions. Methyl donors are able to react with ring nitrogen (N) or oxygen (O) atoms to generate twelve distinct base lesions in DNA (Fu et al., 2012). The proportion of each lesion depends partly on the chemical nature of the methyl donor (e.g., $S_{N1}$ versus $S_{N2}$ nucleophilic substitution) and the DNA substrate (single-stranded versus...
Due to its high nucleophilicity, the predominant methylation adduct produced is N⁷-methylguanine (7meG), which accounts for ~75% of the total methylation lesions in DNA (Beranek, 1990). Although 7meG is relatively harmless to genomic integrity, it is prone to depurination, leading to the formation of potentially toxic and mutagenic abasic sites (Gentil et al., 1992). By contrast, lesions such as N³-methyladenine (3meA) or N¹-methyladenine (1meA) are intrinsically cytotoxic due to their ability to block replicative DNA polymerases (Larson et al., 1985). Translesion polymerases, such as DNA polymerase zeta (ζ), can bypass such lesions but are error prone (Johnson et al., 2007). The presence of 3meA can even result in the initiation of methylation-induced homologous recombination in order to bypass this toxic, polymerase blocking lesion (Hendricks et al., 2002). O⁶-methylguanine (O⁶meG) is a particularly mutagenic lesion as it readily mispairs with thymine during replication (Warren et al., 2006). As a result, the frequency of point mutations increases globally in cells with decreased O⁶meG repair (Esteller and Herman, 2004).

Due to the diverse nature of alkylation damage, three main, highly conserved repair pathways have evolved. These are direct demethylation by O⁶-methylguanine DNA methyltransferase (MGMT), base-excision repair (BER), and the AlkB family of enzymes (Sedgwick, 2004). The complex nature of the lesions created by alkylation and the numerous pathways involved in their reversal necessitates careful coordination of the repair pathways to ensure proper reversal of the damage lesions. In the past decade, many studies have revealed critical insights into the regulatory mechanisms that govern alkylation damage repair. These mechanisms include transcriptional and epigenetic regulation of the repair enzymes as well as post-translational and metabolite-mediated control mechanisms. Here, I will discuss repair by MGMT, BER, and the
AlkB family, along with their regulatory mechanisms, many of which have important implications for improving personalized cancer therapy.

1.1.1 MGMT Expression Status
MGMT repairs O-linked lesions such as O\textsuperscript{6}meG. O\textsuperscript{6}meG is induced by many clinically utilized chemotherapeutic agents including temozolamide (TMZ), dacarbazine, streptozotocin, and procarbazine (Gerson, 2002, 2004). MGMT functions to reverse alkylation damage by the direct transfer of a methyl/alkyl group from the base to a cysteine in its active site in an irreversible reaction (Figure 1.1A). Once MGMT has removed an alkyl group, it is ubiquitinated and targeted for degradation by the proteasome (Xu-Welliver and Pegg, 2002). Because this reaction is not enzymatic, the capacity for repair by MGMT is directly related to the abundance of available MGMT protein. Consequently, increased MGMT expression provides resistance to TMZ and similar alkylating agents by allowing for increased removal of the O\textsuperscript{6}meG lesion (Christmann et al., 2011; Gerson, 2004). Thus, treatment with TMZ and similar agents is more effective for tumors that are deficient in MGMT expression. Methylation at discrete regions within CpG islands of a given gene promoter, an epigenetic marker commonly seen in human cancers, can result in gene silencing (Esteller et al., 1999). Loss of MGMT expression in tumors can be attributed to the methylation and inactivation of the MGMT promoter (Weller et al., 2010). As such, methylation of the MGMT promoter in glioma is a useful predictor of the responsiveness of the tumor to alkylating agents (Esteller et al., 2000). As a chemotherapeutic approach to cancer, numerous strategies have been developed to prevent the repair of O\textsuperscript{6}meG lesions either by depleting or inhibiting MGMT. MGMT activity can be successfully blocked by the addition of free guanine base derivatives, with alkyl groups at the O\textsuperscript{6} position, which act as pseudo-substrates leading to MGMT depletion (Kreklau et al., 1999). Two of the most promising
Figure 1.1. Pathways of Methylation/Alkylation Damage Reversal. (A) Direct reversal of O-linked lesions by methylguanine methyltransferase (MGMT). The methyl group is transferred to a catalytic cysteine, after which MGMT is ubiquitinated and targeted to the proteasome. (B) AlkB-mediated demethylation of N-linked lesions. Initial oxidation of the methyl moiety leads to its hydrolysis to formaldehyde. (C) Repair of N-linked lesions by base excision repair (BER).
MGMT-specific drugs are O\textsuperscript{6}-benzylguanine (BG) and O\textsuperscript{6}-(4-bromothenyl)guanine (PaTrin, PaTrin-2, lomeguatrib) (Dolan et al., 1990; McMurry, 2007; Turriziani et al., 2006). It remains to be seen whether these agents have clinical efficacy against tumors or whether they merely increase the toxicity of alkylating agents.

1.1.2 AlkB Family of Dealkylases
The AlkB family of demethylases repair N-linked adducts occurring on the Watson-Crick base-pairing interface, such as 1meA and N\textsuperscript{3}-methylcytosine (3meC) lesions (Fu et al., 2012). Two of the nine human AlkB members, ALKBH2 and ALKBH3, directly repair these lesions in DNA by an iron and 2-oxoglutarate (2-OG)-dependent oxidative demethylation reaction that reverts the base to its unmodified state by an error free mechanism (Figure 1.1B) (Duncan et al., 2002). The reversal reaction produces succinate and carbon dioxide, with the methyl group being released as formaldehyde (Falnes et al., 2002; Trewick et al., 2002). This reversal of DNA alkylation damage by the ALKBH proteins prevents the accumulation of replication blocks resulting from 1meA and 3meC lesions (Shrivastav et al., 2010).

1.1.3 Coordination of the Steps within Base Excision Repair
The predominant N-linked methylation lesion, 3meA, is repaired by BER. BER operates through a multistep mechanism that repairs the damaged base by the recognition and removal of a single nucleotide (short patch BER) or a small stretch of nucleotides (long patch BER) (Figure 1.1C) (Robertson et al., 2009). Both pathways are initiated by removal of the aberrant base by a DNA glycosylase. While there are 11 distinct mammalian glycosylases (Svilar et al., 2011), the predominant glycosylase relevant for alkylation repair is alkyladenine glycosylase (AAG; also known as methylpurine glycosylase or MPG). The removal of the base results in the formation of an abasic site. Next, the DNA backbone is nicked by the AP endonuclease APE1. Short patch
BER repair is completed by gap filling with DNA polymerase β (Polβ) in association with its scaffolding partner XRCC1 and ligation with DNA ligase I or III (LIG 1/3). While not essential for BER, poly(ADP-ribose)-polymerase (PARP) may serve to facilitate recruitment of certain BER factors (Fortini and Dogliotti, 2007). Long patch BER repair is completed by gap filling with DNA polymerase δ/ε (in proliferating cells) or Polβ, removal of the small stretch of bases by flap endonuclease 1 (FEN1)/proliferating cell nuclear antigen (PCNA), and ligation with LIG1 (Fortini and Dogliotti, 2007).

Unlike the direct reversal mechanisms, the multi-step nature of BER creates several repair intermediates for each alkylated base that is repaired (Figure 1.1C). These include an abasic site, 5’-deoxyribose phosphate, and a single-stranded break (SSB), all of which are potentially cytotoxic and mutagenic lesions (Almeida and Sobol, 2007). Therefore, the steps in BER need to be tightly coordinated with one another to avoid the production and accumulation of these undesirable intermediates. Genetic studies in numerous systems, including yeast, human cells, and mouse transgenic models, have demonstrated that increased expression of individual BER factors can be highly detrimental due to loss of this coordination (Calvo et al., 2013; Ebrahimkhani et al., 2014; Klapacz et al., 2010; Meira et al., 2009; Simonelli et al., 2016; Sobol et al., 2003a; Tang et al., 2010). For example, overexpression of AAG increases cellular sensitivity to the alkylating agent methyl-methane sulfonate (MMS) (Tang et al., 2010). AAG overexpression results in higher rates of frameshift mutagenesis and microsatellite instability. This is due to an increase in abasic site production and an increase in DNA breaks which trigger cell death mediated by PARP1 hyperactivation (Tang et al., 2010). Hypersensitivity to alkylating agents can also be observed in cells where apurinic/apyrimidinic AP endonuclease (APE1) is inhibited, or upon reduced expression of DNA Polβ (Tang et al., 2011). AP site repair can be
prevented with the DNA modifying agent methoxyamine which chemically modifies the AP site and blocks further BER mediated repair, sensitizing cells to alkylating agents (Liu et al., 1999). Overexpression of Polβ prevents the sensitization effect of methoxyamine, suggesting that Polβ expression could be an important metric for determining the effectiveness of methoxyamine as a chemosensitizer (Tang et al., 2011). Loss of AAG in PolB-deficient mouse cells rescues alkylation hypersensitivity which strongly suggests that this phenotype is due to the absence of downstream processing of AAG-induced abasic sites (Sobol et al., 2003a; Sobol et al., 2003b). Deficiency of XRCC1, a scaffold factor involved in BER, also causes alkylation damage sensitivity downstream of the initial steps in BER and results in SSB accumulation (Thompson et al., 1990). The rapid and coordinated assembly of the many BER repair factors during alkylation damage is crucial for the proper functioning of BER.

Poly-ADP-ribosylation has been proposed to be a post-translational modification that promotes the assembly of BER factors downstream of APE1-induced incisions (Almeida and Sobol, 2007). PARP binds to SSBs, where it is activated, and adds poly(ADP-ribose) (PAR) chains to itself and other BER factors. This activation promotes the recruitment of XRCC1, Polβ, and DNA ligase III (Masson et al., 1998). While poly-ADP-ribosylation is not absolutely required for BER, Parp1−/− mice are sensitive to alkylating agents (de Murcia et al., 1997). It was suggested as early as 1977 that PARP inhibitors could be useful for increasing the sensitization of tumors to chemotherapy (Smulson et al., 1977). PARP inhibitors must be carefully integrated into chemotherapy as certain tumors already have an imbalance in BER, exemplified by mutations in Polβ commonly seen in colorectal and other cancers (Nemec et al., 2012; Wallace et al., 2012). Reduced Polβ activity sensitizes tumors to alkylating agents. Inhibition of PARP in this context largely provides short-term rescue of the induced cellular necrosis, suggesting that PARylation
also functions to signal cell death when a BER intermediate accumulates (Tang et al., 2010). However, this imbalance in BER that produces an elevation of PARP1 signaling also promotes long-term sensitization to PARP inhibitors and likely poly(ADP-ribose) glycohydrolase (PARG) inhibitors (Horton et al., 2014; Murai et al., 2012; Prasad et al., 2014). Therefore, identifying a preexisting BER imbalance within a tumor may be critical for determining whether or not PARP inhibition will be beneficial in combination with alkylating chemotherapy. Further, targeting PARG, which removes PAR chains from substrates, could be an alternative option for treating tumors with an intrinsic BER imbalance (Nakadate et al., 2013; Tang et al., 2011). Regardless, knowing the BER status of a given tumor will provide important insight for chemosensitizing strategies.

Importantly, ubiquitination is also a key regulator of the steps of BER. Polβ is ubiquitinated on K206/K244 when not bound to XRCC1 in a process that is regulated by the cell cycle and DNA damage (Fang et al., 2014). In turn, XRCC1 is then a substrate for either HSP90 or the E3 ligase CHIP. It is interesting to speculate that BER may also be targeted (e.g., in homologous recombination defective tumors) via selective regulation of ubiquitination to induce a loss of Polβ, XRCC1, or both of these critical BER proteins.

1.1.4 Cooperation between Base Excision Repair and the AlkB Dealkylases

The existence of multiple alkylation damage repair pathways provides potential redundancy to ensure repair even if one pathway is defective. The AlkB homologues ALKBH2 and ALKBH3 can repair a similar range of lesions, although ALKBH2 prefers to repair lesions in double-stranded DNA (dsDNA) and ALKBH3 prefers to repair lesions in single-stranded substrates (Aas et al., 2003). The association of ALKBH3 with the ASCC3 DNA helicase may expand the substrate range of ALKBH3 to include dsDNA (Dango et al., 2011). Alkbh2−/− mice accumulate
spontaneous genomic 1meA lesions while Alkbh3−/− mice do not have this phenotype, suggesting that ALKBH2 is the major demethylase for endogenous 1meA (Ringvoll et al., 2006). Subsequent studies showed that Alkbh2−/− Alkbh3−/− double-knockout (KO) mice are more susceptible to alkylation-induced tumor development relative to Alkbh2−/− mice, indicating that both enzymes play an essential role in alkylation resistance, albeit to different degrees (Calvo et al., 2012). This study also demonstrated that Aag−/− Alkbh2−/− Alkbh3−/− triple KO mice are highly sensitive to the inflammatory agent dextran sodium sulfate (DSS), which induces exocyclic ethenobase (ε-base) lesion production. Consistently, these triple KO mice accumulated significantly more 1,N6-ethanoadenine (εA) and 1,N2-ethanoguanine (εG) lesions relative to Aag−/− mice. This in vivo work supports previous studies that both BER and the AlkB demethylases are capable of repairing these more complex alkylation lesions in vitro (Delaney et al., 2005; Fu and Samson, 2012; Singer et al., 1992).

AAG and the AlkB proteins function redundantly in their ability to repair certain DNA lesions. However, accumulating evidence suggests that they may compete for some of these adducts. In comparison to other etheno lesions, AAG binds relatively tightly to 3,N4-ethenocytosine (εC) but is incapable of excising this base (Gros et al., 2004; Lingaraju et al., 2011). ALKBH2 demethylates this lesion in vitro, but the presence of AAG inhibits the activity of ALKBH2 in a competitive fashion (Fu and Samson, 2012). Whether this competition is relevant in vivo is unclear. The AAG-εC complex may serve as a signal to recruit ALKBH2 or other relevant factors. However, the AAG-εC complex has the potential to be toxic as it could block the replication or transcription machinery. This complex is reminiscent of the alkyltransferase-like 1 (Atl1) protein in yeast, which is homologous to MGMT but is catalytically inactive (Latypov et al., 2012). By having a high affinity to bulky O6-alkylguanine lesions, Atl1 arrests RNA
polymerase and diverts the repair pathway to transcription-coupled nucleotide excision repair (Latypov et al., 2012). It would be interesting to determine if the recognition of εC by AAG functions similarly as Atl1; it may signal for the recruitment of certain alkylation repair factors, such as ALKBH2 and excluding others, in order to properly match the repair activity with the lesion.

1.1.5 Regulation of Human AlkB Homologues by Ubiquitination
Understanding the regulation of ALKBH2 and ALKBH3 is critical as both are overexpressed in certain tumors, such as prostate adenocarcinoma and non-small cell lung carcinoma (Konishi et al., 2005; Tasaki et al., 2011). While alkylating agents are not typically used for these types of cancer, the differential expression of these proteins may be factors that determine the potential success of alkylation chemotherapy in other tumors. Overexpression of these demethylases may promote resistance to treatment by promoting an increase in alkylation repair (Johannessen et al., 2013). Recent work has demonstrated that both of these enzymes are regulated by the ubiquitin-proteasome system. The enzymes are modified by K48-linked ubiquitination (Zhao et al., 2015), which typically targets proteins for proteasomal degradation (Komander and Rape, 2012). A complex of deubiquitinases positively regulates the dealkylases by countering the ubiquitination. Central to this pathway is the deubiquitinase OTUD4, whose catalytic activity is dispensable for stabilizing ALKBH2/ALKBH3. Instead, OTUD4 functions as scaffolding to link the AlkB homologues to two additional deubiquitinases, USP7 and USP9X (Zhao et al., 2015). In turn, loss of USP7 or USP9X destabilizes ALKBH2 and ALKBH3 resulting in alkylation hypersensitivity. Unlike OTUD4, however, the deubiquitinase activities of USP7 and USP9X are necessary for their function. Interestingly, MGMT stabilization also appears to depend upon the OTUD4/USP7/USP9X pathway (N.M., unpublished observations). This suggests that human
cells may have a master regulatory complex for the enzymes involved in the repair of alkylated DNA. A number of small molecule deubiquitinase inhibitors that target USP7 and USP9X are already available (Chauhan et al., 2012; Kapuria et al., 2010); these inhibitors may provide a novel approach for the chemotherapy sensitization of tumors by targeting multiple repair pathways with a single agent.

1.1.6 Metabolite Regulation of the AlkB Dealkylases
The AlkB dealkylases belong to a large superfamily of Fe\(^{2+}\) and 2-oxoglutarate (α-KG; 2-OG) dependent dioxygenases, which includes the JmjC histone demethylases and the TET family of DNA 5-methylcytosine hydroxylases (Mosammaparast and Shi, 2010; Sundheim et al., 2008). These chromatin-modifying enzymes are susceptible to inhibition by D-2-hydroxyglutarate (D-2HG), which accumulates in tumor cells harboring mutations in isocitrate dehydrogenase (IDH). D-2HG is an oncometabolite that is an antagonist of α-KG (Wang et al., 2015). By inhibiting the demethylation and hydroxylation activities of the JmjC and TET enzymes, respectively, lineage-specific cell differentiation is blocked, contributing to the malignancy of IDH-mutant tumors. D-2HG inhibits the alkylation damage repair activities of ALKBH2 and ALKBH3 in vitro (Wang et al., 2015). Consistently, IDH-mutant cells display significantly slower kinetics for the repair of the AlkB substrate 1meA. These cells also have an accumulation of DNA double-stranded breaks. IDH-mutant cells have significantly increased sensitivity to several alkylating agents, including busulfan and CCNU, which are commonly used to treat chronic myelogenous leukemia and glioma, respectively (Wang et al., 2015). Therefore, the production of D-2HG by IDH-mutant tumors may simultaneously promote tumor development due to its ability to inhibit histone demethylases while simultaneously causing increased susceptibility to certain alkylating agents by inhibiting the AlkB enzymes. Thus, patients harboring IDH-mutant tumors may benefit
from treatment with these alkylating agents but may also require lower doses for tumor responses, reducing their toxic side effects. It will be interesting to determine whether other tumor-associated metabolic changes, such as decreased oxygen tension in the tumor vicinity, are of a sufficient degree to also inhibit the AlkB dioxygenases and therefore increase alkylation sensitivity.

1.1.7 Physiological versus Pathological Methylation
While the canonical AlkB homologues ALKBH2 and ALKBH3 are responsible for the reversal of damaging alkylation adducts, some of their substrates, such as 1meA, may serve physiological functions, at least in RNA, where it associates with the 5’-UTRs of actively translated mRNAs (Dominissini et al., 2016). ALKBH3 demethylation of 1meA in RNA may serve to regulate the physiological function of this RNA modification in addition to protecting RNA from alkylation damage (Aas et al., 2003; Li et al., 2016). Whether cells have the capacity to make the distinction between physiological versus damage-induced 1meA is unknown. The induction of 1meA by an alkylating agent likely induces this modification in an inappropriate region of an mRNA, potentially leading to inhibition of translation or ribosomal miscoding, a phenomenon previously demonstrated for O\textsuperscript{6}meG in mRNA (Hudson and Zaher, 2015). We term this blurring between what is considered damage-induced methylation versus physiological or epigenetic methylation as ‘epigenetic confusion’ (Figure 1.2A). This may also be relevant for N\textsuperscript{6}-methyladenine (6meA), which was recently shown to exist in the genomes of higher eukaryotes, including human cells (Wu et al., 2016). Previous in vitro studies demonstrated that 6meA is produced from 1meA through the base-catalyzed Dimroth rearrangement (Macon and Wolfenden, 1968). Although this has not yet been shown to occur in vivo, the packaging of DNA into chromatin provides a basic environment, where the presence of lysine and arginine residues in the histones
Figure 1.2. Potential Mechanisms of Alkylation Damage-Induced ‘Epigenetic Confusion’. (A) N’-methyladenine (1meA) is associated with 5’-untranslated regions (UTRs) of actively translated mRNAs. Induction of alkylation damage may hinder translation if 1meA is present in the mRNA-coding region. (B) Formation of N°-methyladenine (6meA) from 1meA through Dimroth rearrangement. In the chromatin environment, residues such as lysine may promote nucleophilic attack of 1meA, leading to a ring-opened intermediate, which rearranges to form 6meA. Given that 6meA may have an epigenetic role in DNA, this conversion may lead to its inappropriate placement and function.
have the potential to promote this isomerization (Figure 1.2B). In cells where 1meA is not efficiently repaired, a fraction of the 6meA may be derived from 1meA *in vivo*. Therefore the rapid recruitment of ALKBH2/ALKBH3 to 1meA for the repair of this adducts would be essential to prevent this potential inter-conversion, which would leave an epigenetic mark where it may not be desirable. At least one mechanism of ALKBH2 recruitment has been reported; a direct interaction between ALKBH2 and PCNA targets this repair protein to replication foci (Fu et al., 2015; Gilljam et al., 2009). This interaction increases during DNA replication, suggesting that ALKBH2 recruitment is a cell cycle regulated process. Whether or not other alkylation repair factors are mobilized specifically in response to alkylation damage is unclear. Certain BER factors, such as XRCC1 and DNA Polβ, form nuclear foci upon damage. This suggests that such a mechanism may exist (Fang et al., 2014). However, whether this response is lesion specific or downstream of single-stranded break formation is unknown. Further investigation is needed to determine the upstream signaling responsible for the recruitment of alkylation damage repair factors.

1.2 The ALKBH3-ASCC Complex

1.2.1 ASCC3 Helicase

Our group previously showed that ALKBH3 forms a complex with ASCC (Activating Signal Cointegrator Complex), which plays an important role in repairing alkylated DNA in certain tumor cells, but appears to be dispensable in non-tumor cells (Dango et al., 2011). ASCC is comprised of three proteins: ASCC1, ASCC2, and the DNA helicase, ASCC3. ASCC3 promotes the dealkylation of DNA by ALKBH3 *in vitro*. The importance of the relationship between ASCC3 and ALKBH3 is seen in tumor cell lines that overexpress ALKBH3; the knockdown of ALKBH3 or ASCC3 in these cells results in a decrease in cell proliferation, an increase in genomic 3meC, and an increase in MMS sensitivity (Dango et al., 2011). Due to the association
between ASCC3 and ALKBH3, as well as the DNA helicase activity of ASCC3, it is believed that ASCC3 and ALKBH3 work in concert such that ASCC3 generates the single-stranded substrate needed for ALKBH3-mediated repair of genomic DNA. Therefore, not only is ALKBH3 important for maintaining genomic stability, but ASCC3 also plays a critical role in promoting ALKBH3-mediated repair.

1.2.2 ASCC1 and ASCC2
An outstanding question in the regulation of ALKBH3 dealkylase activity are the roles of ASCC1 and ASCC2. I hypothesize that ASCC1 and ASCC2 serve as accessory factors for ALKBH3 and modulate its activity through distinct mechanisms, thereby influencing resistance to alkylation damage and cell survival. To this end, I am investigating the impact of ASCC1 and ASCC2 activity and function on the ALKBH3-ASCC complex. Here, I provide evidence that ASCC2, like ALKBH3 and ASCC3, localizes to distinct nuclear foci specifically upon exposure of cells to alkylation agents. These nuclear speckle foci associate with alkylated nucleotides and coincide spatially with components of the splicing machinery (Galganski et al., 2017; Spector and Lamond, 2011). Furthermore, recognition of ubiquitin by ASCC2 is critical for the recruitment of ALKBH3 to sites of damage. ASCC1, however, has a distinct role in the localization of the ALKBH3-ASCC complex. ASCC1 is present at nuclear speckles prior to damage, but leaves the foci in response alkylation. Additionally, loss of ASCC1 significantly increases ASCC3 foci formation during alkylation damage, yet most of these foci lack ASCC2. Finally, I demonstrate that both ASCC1 and ASCC2 promote cell survival after treatment with alkylation agents. Together, these results suggest that ASCC1 and ASCC2 coordinate the recruitment of the ALKBH3-ASCC complex during alkylation damage. The findings herein
contribute significantly to the identification of regulators of the ALKBH3 alkylation damage repair response.
Chapter 2: A Ubiquitin-Dependent Signaling Axis Specific for ALKBH-Mediated DNA Dealkylation Repair


2.1 Abstract
DNA repair is essential to prevent the cytotoxic or mutagenic effects of various types of DNA lesions, which are sensed by distinct pathways to recruit repair factors specific to the damage type. Although biochemical mechanisms for repairing several forms of genomic insults are well understood, the upstream signaling pathways that trigger repair are established for only certain types of damage, such as double-stranded breaks and interstrand crosslinks (Jackson and Durocher, 2013; Sirbu and Cortez, 2013; Zhao et al., 2014). Understanding the upstream signaling events that mediate recognition and repair of DNA alkylation damage is particularly important. Alkylation chemotherapy is one of the most widely used systemic modalities for cancer treatment. Additionally, environmental chemicals may trigger DNA alkylation (Drablos et al., 2004; Fu et al., 2012; Sedgwick et al., 2007). Here we demonstrate that human cells have a previously unrecognized signaling mechanism for sensing damage induced by alkylation. We find that the alkylation repair complex ASCC (Dango et al., 2011) re-localizes to distinct nuclear foci specifically upon exposure of cells to alkylating agents. These foci associate with alkylated nucleotides and coincide spatially with elongating RNA polymerase II as well as splicing components. Proper recruitment of the repair complex requires recognition of K63-linked polyubiquitin by the CUE (coupling of ubiquitin conjugation to ER degradation) domain of the ASCC2 subunit. Loss of this subunit impedes alkylation adduct repair kinetics and increases
sensitivity to alkylating agents, but not other forms of DNA damage. We identify RING finger protein 113A (RNF113A) as the E3 ligase responsible for upstream ubiquitin signaling in the ASCC pathway. Together, our work reveals a previously unrecognized ubiquitin-dependent pathway induced specifically to repair alkylation damage.

2.2 Introduction

A crucial first step in DNA repair involves the recognition of the damage. This activates signaling pathways that recruit effectors to resolve the lesion. However, whether this ‘sensor-transducer-mediator’ model is generally applicable to pathways dedicated to repairing each distinct type of DNA lesion, such as alkylated lesions, remains unknown. In humans, the alkylation adducts 1-methyladenine (1meA) and 3-methylcytosine (3meC) are repaired by ALKBH2 and ALKBH3 (Fu et al., 2012). Understanding the regulation of these repair proteins in critical as many of the chemotherapeutics that are used to treat cancer induce these and other alkylated lesions.

ALKBH3 was once thought to play a minor role as compared to ALKBH2 in the reversal of 1meA and 3meC. However, there is accumulating evidence that ALKBH3 has an important pathological function. ALKBH3 is overexpressed in both prostate and non-small-cell lung cancer (Konishi et al., 2005; Tasaki et al., 2011). The knockdown of ALKBH3 in these cell lines results in decreased cell proliferation, increased sensitivity to MMS treatment, and increased 3meC in genomic DNA (Dango et al., 2011). This indicates that ALKBH3 is critical for maintaining genomic stability in specific tumor cells.

One of the outstanding questions about repair by the ALKBH family of proteins is their mechanism of recruitment to sites of alkylation damage. ALKBH3 has been shown to interact with ASCC2 and ASCC3 (Dango et al., 2011). The ASCC3 helicase is believed to able to
promote repair by ALKBH3 by generating the single-stranded substrate that ALKBH3 prefers (Chen et al., 2010; Monsen et al., 2010). Here, we will investigate the function of the uncharacterized ASCC2 protein. We find that recruitment of the ALKBH3-ASCC repair complex to sites of alkylation damage requires recognition of K63-linked polyubiquitin by the CUE domain of the ASCC2 subunit. Additionally, the ubiquitination of Brr2 by the E3 ubiquitin ligase RNF113A promotes the recruitment of ASCC2 to sites of alkylation induced damage. Together, our data suggest a ubiquitin-dependent recruitment pathway for the repair of alkylation induced adducts by ALKBH3.

2.3 Results
2.3.1 The ASCC Complex Forms Foci upon Alkylation Damage
Previous studies established that the dealkylating enzyme ALKBH3 functions in concert with the ASCC3 helicase (Dango et al., 2011). We tested the subcellular localization of ASCC3 upon exposure to various DNA damaging agents. Endogenous ASCC3 formed nuclear foci upon treatment of U2OS cells with the alkylating agent methyl methanesulfonate (MMS). Strikingly, other types of DNA damaging agents did not significantly induce ASCC3 foci (Figure 2.1A-B). All of the genotoxins that were used induced pH2A.X foci, indicating that the doses used were sufficient to induce DNA damage signaling. ASCC3 foci were also observed upon treatment with busulfan, CCNU, and temozolomide (TMZ), alkylating agents used clinically in the treatment of various tumors, further confirming the role of ASCC3 in the repair of alkyl adducts (Figure 2.1C) (Wick and Platten, 2014). The ASCC2 subunit of the ASCC complex also formed foci specifically after treatment with MMS (Figure 2.1D). Consistent with their known physical association (Dango et al., 2011; Jung et al., 2002), endogenous ASCC3 foci co-localized with both HA-ASCC2 and HA-ALKBH3 foci upon MMS treatment (Figure 2.2A). Conversely, factors from two other alkylation damage repair pathways, methylguanine methyltransferase
Figure 2.1. The ASCC Complex Forms Foci upon Alkylation Damage. (A) Images of ASCC3 and pH2A.X immunofluorescence after treatment with damaging agents. CPT, camptothecin; HU, hydroxyurea. (B) ASCC3 foci quantification from (A). n = 3 biological replicates and error bars indicate ± S.D. of the mean; two-tailed t-test, * = p < 0.001. Bleo, bleomycin. (C) Images of U2OS cells after treatment with the alkylating agents busulfan (4 mM), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU; 100 μM) or temozolamide (TMZ; 1.0 mM). n = 2 biological replicates. Numbers indicate the mean percentage of cells expressing ≥ 5 ASCC3 foci. (D) Immunofluorescence of HA-ASCC2 expressing cells after exposure to the indicated damaging agents. n = 3 biological replicates. Scale bar, 10 μM.
Figure 2.2. Localization of ASCC Complex and Other Alkylation Repair Factors. (A) Images of cells expressing HA-ASCC2 or HA-ALKBH3 after MMS treatment. n = 2. (B) Immunofluorescence of cells expressing HA-ALKBH2, HA-MGMT, or HA-AAG upon MMS treatment. (C) Quantification of ASCC3 co-localization from (B). Error bars indicate ± S.D. of the mean.
(MGMT) and the base-excision repair glycosylase AAG, showed minimal co-localization with ASCC3 after alkylation damage (Figure 2.2B-C).

To ascertain whether or not the ASCC complex is recruited to regions of the nucleus that have alkylation damage, we performed a proximity ligation assay (PLA) in U2OS cells. We found that a specific nuclear PLA signal between 1meA and ASCC3 is induced upon MMS damage (Figure 2.3A-B). This signal was significantly reduced in the U2OS CRISPR-Cas9 generated ASCC3 knockout (KO) cells (Figure 2.3A-C). Additionally, ASCC foci did not co-localize with either pH2A.X or 53BP1 (Figure 2.3D). This demonstrates that the ALKBH-ASCC foci are distinct from double-stranded break-induced foci.

We took an unbiased proteomic approach to identify additional factors associated with ASCC foci in response to alkylation damage. Flag-HA-ASCC2 was purified from HeLa-S nuclear extract by tandem affinity purification (Figure 2.4A) (Biancalana et al., 2004) with and without treatment with MMS. The subsequent mass spectrometry (MS) analysis of the ASCC2-associated proteins revealed the constitutive association of ASCC3 and ASCC1 with ASCC2. ASCC2 also associated with many spliceosomal components and basal transcription factors (Figure 2.4B). These factors, including BRR2, PRP8, and TFII-I, had two- to threefold higher total peptide numbers in cells exposed to MMS than in the untreated sample. This suggests an increase in association between these factors and the ASCC complex in response to alkylation-induced damage. In conformation of the MS results, focused immunofluorescence studies revealed that ASCC components co-localized with BRR2 and PRP8 upon alkylation damage (Figure 2.4C-D).
Figure 2.3. ASCC Foci Co-Localize with 1meA. (A) PLA images in control or MMS treated cells using N1-methyladenosine (1meA) and ASCC3 antibodies. n = 3 biological replicates. (B) PLA quantification of (A). n = 3 biological replicates. Error bars indicate ± S.D. of the mean. (C) ASCC3 knockout (KO) cells were generated using CRISPR/Cas9 technology. Lysates were analyzed by Western blotting. Clone #10 was verified to be a knockout by deep sequencing. (D) Images of U2OS or U2OS cells expressing the indicated vectors after MMS treatment. n = 3 biological replicates.
Figure 2.4. ASCC2 Co-Localizes with Spliceosomal Components and Basal Transcription Factors. (A) Silver staining of the Flag-HA-ASCC2 complex purified from HeLa-S nuclear extract. (B) Tagged ASCC2 was purified with or without MMS and analyzed by mass spectrometry. Peptide numbers for identified proteins were plotted for each condition. Expanded view is shown on the right. (C) Immunofluorescence of HA-ASCC2-expressing cells treated with MMS. (D) Quantification of MMS-induced co-localizations of HA-ASCC2 foci from (C). $n = 3$ biological replicates. Error bars indicate ± S.D. of the mean.
Due to the association of ASCC factors with spliceosomal components, we next tested if ASCC3 was able to bind ssRNA. A recombinant, N-terminal truncation of ASCC3 (NΔ-ASCC3; residues 401-2202) bound to ssRNA in vitro (Figure 2.5A). Consistent with the notion that ASCC3 binds RNA, RNase treatment before processing for immunofluorescence significantly reduced ASCC3 foci formation (Figure 2.5B). Together these data indicate not only that the ALKBH3-ASCC complex forms foci in response to alkylation damage, but also that these foci form preferentially at sites of active transcription and splicing. This is consistent with recent ChIP-Seq studies on ALKBH3 which suggest an enriched presence of this dealkylase at highly active promoters (Liefke et al., 2015).

### 2.3.2 ASCC2 Binds to K63-Linked Ubiquitin Chains via its CUE Domain

Although recruitment of certain repair complexes is dependent on specific upstream signaling kinases (Jackson and Durocher, 2013; Sirbu and Cortez, 2013; Zhao et al., 2014), this is not the case for the recruitment of the ASCC complex. Inhibition of ATM (ataxia-telangiectasia mutated) moderately increased ASCC3 foci formation. Inhibition of ATR (ataxia-telangiectasia and Rad3 related) made no impact on foci formation (Figure 2.6A). We found that HA-ASCC2 foci co-localized with polyubiquitin, suggesting that ubiquitin signaling could be responsible for the recruitment of this repair complex to sites of alkylation damage (Figure 2.6B). Analysis of the ASCC2 protein sequence revealed a highly conserved CUE domain (residues 467-509) which belongs to the ubiquitin-binding domain superfamily (Komander and Rape, 2012) (Figure 2.7A). Recombinant His-ASCC2 bound K63-, but not K48-linked ubiquitin chains in vitro (Figure 2.7B). K63-linked ubiquitination has been shown to be important for the innate immune response whereas K48-linked ubiquitination is a canonical signal that targets protein for degradation by
Figure 2.5. ASCC3 Binds RNA. (A) Biotinylated RNAs (20-mer, 35-mer, or 50-mer) were immobilized and tested for binding to recombinant His-(NΔ)-ASCC3. n = 2 independent experiments. (B) U2OS cells were treated with MMS and processed for immunofluorescence with or without initial incubation with RNase A (50 nM). Numbers indicate the percentage of cells expressing ≥ 5 ASCC3 foci. n = 3 biological replicates. Mean ± S.D.
Figure 2.6. ASCC2 Co-Localizes with Polyubiquitin. (A) Immunofluorescence of endogenous ASCC3 foci. Numbers indicate the percentage of cells expressing ≥ 5ASCC3 foci. n = 2 biological replicates. (B) Immunofluorescence of HA-ASCC2 and FK2 in cells after MMS treatment. n = 3 biological replicates. Mean ± S.D.
Figure 2.7. ASCC2 Binds to K63-Linked Ubiquitin Chains. (A) ASCC2 sequence alignment. (B) His-ASCC2 was immobilized and assessed for binding to K48-Ub₂₋₇ (left) or K63-Ub₃₋₇ (right). ALKBH3 and gp78-CUE served as controls. Bound material was analyzed by Western blotting or Coomassie Brilliant Blue (CBB). n = 3 independent experiments. (C) Immunofluorescence of HA-ASCC2 cells and K63-Ub (top) or K48-Ub (bottom) after MMS treatment. n = 2 independent experiments.
the proteasome (Akutsu et al., 2016). Furthermore, ASCC2 co-localized with K63-, but not K48-linked ubiquitin foci upon MMS damage (Figure 2.7C).

We introduced point mutations in the ASCC2 CUE domain at residues predicted to be critical for ubiquitin recognition (Figure 2.8A). These mutants, ASCC2(L506A) and ASCC2(LL478-9AA), abrogated K63-linked ubiquitin binding in vitro. The ASCC2(P498A) mutation, which was not predicted to be important for ubiquitin recognition, bound to K63-Ub similarly to the ASCC2 wild-type control (Figure 2.8B). Notably, both of the ASCC2 mutants that abrogate ubiquitin binding also showed significantly reduced foci formation upon MMS treatment (Figure 2.8C). Thus, not only does ASCC2 bind to K63-Ub, but the binding of ASCC2 to K63-Ub is necessary for foci formation after alkylation damage. Together, these data support the notion that ASCC2 is recruited to sites of alkylation damage in a manner that is dependent upon ubiquitin binding.

2.3.3 ASCC2 is Critical for ASCC3-ALKBH3 Recruitment and Alkylation Resistance

We reasoned that ASCC2 could act as an intermediary subunit required for the recruitment of the additional components of the ALKBH3-ASCC complex. To test this, we generated ASCC2 knockout cells using CRISPR-Cas9 (Figure 2.9A). Two independent ASCC2 knockout clones showed a significant reduction in endogenous ASCC3 foci formation upon MMS treatment (Figure 2.9B-C). HA-ALKBH3 foci were also diminished in the ASCC2 KO cells (Figure 2.9D). Thus, ASCC2 promotes the recruitment of both ASCC3 and ALKBH3 to foci. Consistent with the role of ASCC2 in the recruitment of alkylation damage repair factors to sites of alkylation damage, ASCC2-deficient PC-3 cells were hypersensitive to treatment with MMS, but not camptothecin or bleomycin. This was assayed by both MTS survival and colony formation assays (Figure 2.10).
Figure 2.8. ASCC2 CUE Domain is Important for Binding K63-Linked Ubiquitin Chains. (A) Interaction model between ubiquitin and the CUE domain of ASCC2 (PDB accession number 2D10). The positions of four residues (L478, L479, P498, and L506) are shown. (B) Binding assays were performed with K63-Ub:: using His-tagged ASCC2 WT or indicated ASCC2 mutant. n = 3 independent experiments. (C) Immunofluorescence images of MMS induced foci in cells expressing various forms of HA-ASCC2. Numbers indicate the percentage of cells expressing ≥ 10 HA-ASCC2 foci. n = 3 biological replicates. Mean ± S.D.
Figure 2.9. ASCC2 is Critical for ASCC3-ALKBH3 Recruitment. (A) U2OS and PC-3 ASCC2 KO cells were generated using CRISPR/Cas9 technology and verified by deep sequencing. Whole cell lysates of the parental and KO cells were analyzed by Western blotting. (B) MMS-induced ASCC3 foci were assessed in WT and ASCC2 KO cells. (C) Quantification of ASCC3 foci from (B). n = 3 biological replicates. Error bars indicate ± S.D. of the mean. * = p < 0.001. (D) MMS-induced HA-ALKBH3 foci were assessed in WT and ASCC2 KO cells. Numbers indicate the percentage of cells expressing ≥ 5 foci. n = 2 biological replicates. Mean ± S.D.
Figure 2.10. ASCC2 KO Cells are Hypersensitive to Treatment with Alkylating Agents. (A, B, C) Sensitivity of WT or ASCC2 knockout cells to (A) MMS, (B) camptothecin, or (C) bleomycin was assessed by MTS assay. n = 5 biological replicates. Mean ± S.D. (D, E) Sensitivity of WT and ASCC2 knockout cells to (D) MMS or (E) camptothecin was assessed by clonogenic survival assay. n = 4 biological replicates. Mean ± S.D.
Next, we reconstituted ASCC2 knockout cells by expressing wild-type and mutant versions of ASCC2 in order to rescue both the foci formation and MTS assays. Wild-type HA-ASCC2 restored MMS-induced ASCC3 (Figure 2.11A-B) and HA-ALKBH3 foci formation (Figure 2.11C-D). The ASCC2 (L506A) CUE domain mutant, which abrogated binding to K63-Ub, was not able to rescue foci formation (Figure 2.11). This further confirms the importance of ubiquitin binding in the recruitment of not only ASCC2 but also the entire ALKBH3-ASCC repair complex. Similarly, wild-type, but not ASCC2(L506A), rescued the MMS sensitivity of ASCC2 knockout cells (Figure 2.12).

We confirmed that the L506A mutation did not impact the binding interaction between ASCC2 and ASCC3; HA-tagged wild-type and ASCC2(L506A) equally co-immunoprecipitated ASCC3 (Figure 2.13A). Further, His-ASCC3 bound to both immobilized Flag-ASCC2 and Flag-ALKBH3, although the interaction with ALKBH3 appears to be weaker (Figure 2.13B). Deletion of the ASCC3 N-terminus (ASCC3-NΔ; residues 401-2202) abrogated the ASCC2-ASCC3 interaction. Conversely, ALKBH3 bound to ASCC3-NΔ (Figure 2.13C). To further investigate the binding conformation of the complex components, binding between ASCC2 and ALKBH3 was assessed. No interaction was detected between ASCC2 and recombinant ALKBH3 (Figure 2.13D). The C-terminal domain of ASCC3 was used as a positive control (ASCC3-C; residues 1301-2202). ASCC2 therefore appears to bridge ASCC3 and K63-linked ubiquitin chains. ALKBH3 is indirectly recruited to foci by ASCC2 through its interaction with ASCC3 (Figure 2.13E).

### 2.3.4 RNF113A Ubiquitination Recruits the ASCC Complex
We next endeavored to determine the E3 ubiquitin ligase that is functioning in this alkylation damage repair pathway. To identify this E3 ligase, we performed a screen using a custom library
Figure 2.11. See next page for caption.
Figure 2.11. Characterization of ASCC2 KO Cells. (A) Images of WT or ASCC2 knockout cells expressing indicated vectors upon MMS treatment. (B) Quantification of HA-ALKBH3 foci from (A). n = 3 biological replicates. Error bars indicate ± S.D. of the mean. * = p < 0.001, # = p < 0.05. (C) Images of WT or ASCC2 knockout cells expressing the indicated vectors after MMS exposure. (D) Quantification of ASCC3 foci from (C). n = 2 independent experiments. Error bars indicate ± S.D. of the mean. (E) Whole-cell lysates were collected and expression was analyzed by Western blotting. n = 2 independent experiments.
Figure 2.12. WT ASCC2 Rescues Hypersensitivity to Alkylating Agents. WT or ASCC2 knockout cells expressing indicated vectors were assessed for sensitivity to MMS using the MTS assay. n = 5 technical replicates. Error bars indicate ± S.D. of the mean.
Figure 2.13. ASCC2 Coordinates ASCC-ALKBH3 Complex Recruitment during Alkylation Damage. (A) Immunoprecipitation of HA-ASCC2 or HA-ASCC2 L506A was performed and analyzed by Western blotting as shown. n = 2 biological replicates. (B) Flag-ASCC2 or Flag-ALKBH3 was immobilized and tested for binding to full-length (FL) His-ASCC3. n = 2 independent experiments. (C) Flag-ASCC2 or Flag-ALKBH3 was immobilized and tested for binding to N-terminally deleted His-ASCC3 (His-ASCC3-ΔN). n = 2 independent experiments. (D) Flag-ALKBH3 was immobilized and tested for binding to His-ASCC2, with His-ASCC3-C (C terminus of ASCC3) serving as a positive control. n = 2 independent experiments. (E) ASCC-ALKBH3 complex model.
of short hairpin RNAs (shRNAs) that target UBC13-interacting E3 ligases or other ligases implicated in DNA repair. This library was chosen because UBC13 is known to be a major E2 ubiquitin ligase responsible for the formation of K63-linked ubiquitin chains. Additionally, UBC13 has been implicated in DNA damage response pathways (Thorslund et al., 2015; Unk et al., 2006; Zhao et al., 2007). The screen identified RNF113A as a potential candidate, with three distinct shRNAs reducing HA-ASCC2 foci formation (data not shown). We confirmed that these shRNAs attenuated both RNF113A protein levels and HA-ASCC2 foci formation after alkylation damage (Figure 2.14A-B). Importantly, MMS-induced ASCC2 foci co-localized with RNF113A foci (Figure 2.14C). In the absence of any damage, RNF113A co-localized with PRP8 and BRR2 (Figure 2.14D). This is consistent with previous studies which suggest that RNF113A may serve as a component of the spliceosome (Hegele et al., 2012).

To uncover the relevant RNF113A substrate, we combined our initial proteomics screen which looked for ASCC2 interacting proteins that were enriched by treatment with MMS (Figure 2.4B) with a second proteomics screen which looked for ASCC2 interacting proteins which interacted preferentially with wild-type ASCC2 relative to ASCC2(L506A) (Figure 2.15). There were 295 proteins that were enriched in both datasets. Eight of these had been shown to be ubiquitinated by UBC13 (Thorslund et al., 2015). Of these eight proteins, BRR2 was the most obvious candidate, as it co-localized with both RNF113A and ASCC components by immunofluorescence. BRR2 co-immunoprecipitated RNF113A in a manner dependent upon the N-terminal domain of RNF113A (Figure 2.16A). Deletion analysis revealed that the RNF113A N-terminus was also critical for its co-localization with the spliceosomal component PRP8 (Figure 2.16B-C). Knockdown of BRR2, or its partner PRP8 (Hegele et al., 2012), significantly
Figure 2.14. Identification of the RNF113A E3 Ligase. (A) MMS-induced foci in U2OS cells expressing indicated shRNAs. n = 3 technical replicates. Numbers indicate mean. (B) Whole-cell lysates of U2OS cells infected with the indicated shRNAs were analyzed by Western blotting. Asterisk indicates a non-specific band in the RNF113A blot. n = 2 independent experiments. (C) Localization of Flag-ASCC2 and HA-RNF113A after MMS treatment. n = 3 biological replicates. (D) Immunofluorescence of cells expressing Flag-RNF113A without damage treatment. n = 3 biological replicates.
Figure 2.15. Proteomic Analysis of Relevant RNF113A Substrates. ASCC2 interactome analysis. UBC13 substrates were previously described by Sowa et al., 2009.
Figure 2.16. Functional Characterization of RNF113A. (A) HA-RNF113A deletions were immunoprecipitated to analyze BRR2 interaction. n = 3 independent experiments. CΔ, C-terminal domain deletion; NΔ, N-terminal domain deletion; ΔZF, zinc finger domain deletion. (B) Schematic of human RNF113A and its domain structure. The three deletion constructs used for localization analysis are also shown. (C) Images of cells expressing WT or the indicated HA-RNF113A deletion constructs. Numbers indicate quantification of co-localization between each RNF113A construct and PRP8. n = 3 biological replicates. Mean ± S.D.
reduced ASCC3 foci formation upon MMS damage (Figure 2.17). Thus, BRR2 represents a physiological substrate for RNF113A in this alkylation repair pathway.

2.4 Discussion
Our results provide the first evidence for an alkylation-specific damage response in human cells. The ASCC complex acts as a major node in this pathway, sensing ubiquitin-dependent signaling (via ASCC2) and concomitantly recruiting alkylation repair enzymes (ALKBH3 and ASCC3). As such, ASCC2 serves as an adaptor, and may be analogous to Rap80, which recruits the BRCA1 complex to chromatin during the double-stranded break response (Jackson and Durocher, 2013). Indeed, Rap80 recognizes non-proteosomal ubiquitin chains produced by the upstream RNF8/RNF168 E3 ubiquitin ligases before BRCA1 recruitment. Here, RNF113A functions as the E3 ligase that transduces the alkylation damage signal. How alkylation damage uniquely activates RNF113A to recruit ASCC2 versus other repair complexes will be an important question for future studies. RNF113A contains a CCCH-type zinc finger, a motif known to bind RNA. Since RNA is also modified by exposure to alkylating agents, it is possible that damaged RNA serves as the initial signal to activate DNA alkylation repair. As our work strongly suggests the presence of a cellular sensor specific for alkylation damage in human cells, it may be possible to target this pathway to improve tumor responses to conventional chemotherapy. Future studies will undoubtedly clarify these questions about the upstream signals for this novel damage signaling pathway.

2.5 Materials and Methods
Data reporting: No statistical methods were used to predetermine sample size. The experiments were not randomized and the investigators were not blinded to allocation during experiments and outcome assessment.
Figure 2.17. RNF113A Ubiquitination Recruits the ASCC Complex. (A) Images of U2OS cells expressing indicated shRNAs. (B) Quantification of ASCC3 foci from (A). n = 3 biological replicates. Error bars indicate ± S.D. of the mean. # = p < 0.001. (C) Western blotting analysis of U2OS cells expressing the indicated shRNAs used for immunofluorescence analysis in (A). n = 2 independent experiments.
**Plasmids:** Human ALKBH3, ASCC2, ASCC3, RNF113A, BRR2, and gp78 cDNAs were isolated as previously described (Dango et al., 2011). For mammalian cell expression, cDNAs were subcloned into pHAGE-CMV-3xHA, pHAGE-CMV-Flag, or pMSCV-Flag-HA as needed by Gateway recombination (Sowa et al., 2009). The cDNA for PCNA (a gift from Z. You, Washington University) was subcloned into pHAGE-CMV-GFP. For recombinant protein expression, cDNAs were subcloned into pGEX-4T1, pET28a-Flag, or pDEST10. All constructs derived by PCR, including deletions and point mutations, were confirmed by Sanger sequencing.

**Cell culture and cell survival assays:** U2OS, PC-3, HeLa-S, and HEK293T cells (originally from the American Type Culture Collection) were cultured and maintained as previously described (Zhao et al., 2015). Cells were tested for mycoplasma at the Washington University Genome Engineering and iPSC Center, and were authenticated using the ATCC human STR profiling services. Preparations of viruses, transfection, and viral transduction were performed as described previously (Zhao et al., 2015). For rescue experiments of knockout cell foci cells were transduced with the pHAGE-CMV-3xHA or pHAGE-CMV-Flag lentiviral rescue vector. For rescue experiments of knockout-cell MMS sensitivity, cells were transduced with the pMSCV-Flag-HA retroviral rescue vector. For survival assays of DNA damaging agent using PC-3 cells, 10,000 cells per well were cultured overnight in 96-well plates in 100 μl media. Cells were then exposed to medium containing the indicated concentration of MMS (Sigma) for 24 h at 37 °C. The media were then replaced with normal media, and cell viability was assessed using the MTS assay (Promega) 72 h after initial damaging agent exposure. For experiments involving camptothecin, or bleomycin (both purchased from Sigma), cells were exposed to medium containing the indicated concentration of the damaging agent in culture medium for 72 h at
37 °C. Viability was then processed by MTS assay as above. All MTS-based survival experiments were done in quintuplicate.

**CRISPR-Cas9-mediated knockouts:** U2OS and PC-3 knockout cells were created using CRISPR-Cas9 genome editing at the Genome Engineering and iPSC Center (GEiC) at Washington University School of Medicine (St. Louis, Missouri, USA). PC-3 ASCC2 and ASCC3 knockout clones were initially assessed by deep sequencing and confirmed by Western analysis. All other knockout clones were isolated and confirmed by Western analysis. The gRNA sequences used to generate the knockout cell line were as follows: ASCC2, 5′-GCCAAGTTACTACAGTGACCTGG-3′; ASCC3, 5′-ATGGCTTTACCTCGTCTCACAGG-3′.

**Immunofluorescence microscopy:** All immunofluorescence microscopy was performed as previously described (Zhao et al., 2015) with minor modifications. After treatment with the indicated damaging agent in complete medium at 37 °C for 6 h (500 μM MMS, unless indicated otherwise; 1 μM camptothecin; 10 mM hydroxyurea; 20 μM bleomycin; 5 Gy IR; or 25 J m⁻² ultraviolet light), U2OS cells were extracted with 1× PBS containing 0.2% Triton X-100 and protease inhibitors (Pierce) for 10-20 min on ice before fixation with 3.2% paraformaldehyde. The cells were then washed extensively with immunofluorescence wash buffer (1× PBS, 0.5% NP-40, and 0.02% NaN3), then blocked with immunofluorescence blocking buffer (immunofluorescence wash buffer plus 10% FBS) for at least 30 min. Primary antibodies were diluted in immunofluorescence blocking buffer overnight at 4 °C. After staining with secondary antibodies (conjugated with Alexa Fluor 488 or 594; Millipore) and Hoechst 33342 (Sigma-Aldrich), where indicated, samples were mounted using Prolong Gold mounting medium (Invitrogen). Epifluorescence microscopy was performed on an Olympus fluorescence microscope (BX-53) using an ApoN 60x/1.49 numerical aperture oil immersion lens or an
UPlanS-Apo 100×/1.4 numerical aperture oil immersion lens and cellSens Dimension software. Raw images were exported into Adobe Photoshop, and for any adjustments in image contrast or brightness, the levels function was applied. For foci quantification, at least 100 cells were analyzed in triplicate, unless otherwise indicated.

**Colony formation assay:** Parental or knockout cells were trypsinized, counted, and plated at low density. After overnight incubation, the cells were treated with the indicated doses of MMS or camptothecin for 24 h in complete medium. The cells were incubated for 12-14 days, fixed, and stained with crystal violet. The experiment was performed in quadruplicate for each cell line and drug dose. Colonies were counted and relative survival was normalized to untreated controls.

**In situ PLA:** PLA was performed using a Duolink detection kit (Sigma) following the manufacturer’s instructions with minor modifications. U2OS cells were extracted and fixed as described above. The cells were washed extensively with immunofluorescence wash buffer, then blocked with 1× Duolink blocking solution for 30 min at 37 °C in a pre-warmed humidifier. Primary antibodies were diluted in 1× Duolink antibody diluent and added to samples overnight at 4 °C. Samples were washed with wash buffer A at room temperature. PLA probes anti-rabbit PLUS and anti-mouse MINUS were diluted 1:5 in 1× Duolink antibody diluent, then added to samples and incubated for 1 h at 37 °C. Samples were again washed with wash buffer A at room temperature. Duolink ligation stock and ligase were diluted 1:5 and 1:40 in pure water, respectively, and applied to samples for 30 min at 37 °C. After washing with wash buffer A, Duolink amplification red probe and polymerase were diluted 1:5 and 1:80 in water and applied to samples for 100 min at 37 °C. After a brief wash with wash buffer B, samples were mounted using Duolink mounting medium with DAPI and imaged as above.
**Purification of TAP-ASCC2 complexes and MS/MS analysis:** Affinity purification of ASCC2 was performed as previously described for ALKBH3 with minor modifications (Dango et al., 2011). Briefly, Flag-HA-ASCC2 was stably expressed after transduction of pMSCV-Flag-HA-ASCC2 retrovirus into HeLa-S cells. Nuclear extract was prepared from the stable cell line with or without previous treatment with MMS (400 μM for 6 h), and the ASCC2 complex was purified using anti-Flag (M2) resin (Sigma), followed by purification using anti-HA (F-7) resin (Santa Cruz) in TAP buffer (50 mM Tris-HCl pH 7.9, 100 mM KCl, 5 mM MgCl2, 10% glycerol, 0.1% NP-40, 1 mM DTT, and protease inhibitors). For comparison of wild-type ASCC2 versus ASCC2 L506A, the same method was used, except that both samples were treated with MMS and the HA purification was omitted. After peptide elution, the complexes were TCA precipitated and associated proteins were identified by LC-MS/MS at the Taplin Mass Spectrometry Facility (Harvard Medical School, Boston, Massachusetts, USA) using an LTQ Orbitrap Velos Pro ion-trap mass spectrometer (ThermoFisher) and Sequest software (Eng et al., 1994).

**Protein purification:** Recombinant proteins (ALKBH3, ASCC2, ASCC3, and gp78 CUE) were purified from Rosetta (DE3) or Sf9 cells using an ÄKTA-pure FPLC (GE Healthcare). For His-tagged bacterially expressed proteins, cells were resuspended in His-lysis buffer (50 mM Tris-HCl pH 7.3, 250 mM NaCl, 0.05% Triton X-100, 3 mM β−ME, 30 mM imidazole, and protease inhibitors) and lysed by sonication. After centrifugation and filtration, the extract was loaded onto a HisTrap HP column using a 50 ml Superloop (GE Healthcare). After extensive washing with lysis buffer, the protein was eluted using lysis buffer containing 400 mM imidazole. His-Flag-ALKBH3 was further purified on a Superdex 200 Increase 10/300 GL size-exclusion column. All recombinant proteins were dialyzed into TAP buffer. Flag-tagged RNF113A was
purified from HeLa-S cells by resuspension in Flag-lysis buffer (50 mM Tris-HCl pH 7.9, 150 mM NaCl, 10% glycerol 1.0% Triton X-100, 1 mM DTT, and protease inhibitors) and lysed by sonication. After incubation with Flag resin, the protein was eluted with lysis buffer containing 0.4 mg ml⁻¹ Flag peptide.

**Protein and RNA binding assays:** All *in vitro* binding assays were performed as previously described (Drablos et al., 2004), with minor modifications. Flag (M2) agarose and Ni-NTA agarose beads were pre-blocked with 10% bovine serum albumin. For ubiquitin binding assays, 10 µg of His-ASCC2 or His-ASCC2 mutants were added to each reaction, along with 500 ng of either K48- or K63-Ub₂-7 (Boston Biochem). The indicated proteins were added to 10 µl of beads in a total volume of 100 µl with TAP wash buffer. Reactions were incubated at 4 °C with rotation for 1 h, then washed extensively with TAP wash buffer. A final wash was performed with 1× PBS, and bound material was eluted with 20 µl of Laemmli buffer, analyzed by SDS-PAGE, and stained with Coomassie blue or subjected to western analysis as indicated. For RNA binding experiments, 0.5 nmol of each 5′-biotinylated RNA (50-mer sequence: 5′-UCGAUAGUCUCUCAGACAGCAUGUCCUAAGCAAGCCAGAAUGUCGGCAGCGUC-3′; the 35-mer and 20-mer removed 15 and 30 nucleotides from the 3′ end of the same sequence, respectively) was immobilized on 10 µl streptavidin-agarose beads. To each reaction, 1 µg of His-NΔ-ASCC3 (residues 401-2202) was added in a total volume of 100 µl in TAP wash buffer with RNase inhibitor (Jost et al.). Reactions were incubated at 25 °C with rotation for 30 min, then washed extensively with TAP wash buffer. A final wash was performed with 1× PBS, and bound material was eluted with 10 µl of Laemmli buffer, analyzed by SDS-PAGE and western blotting.
**Immunoprecipitation:** Immunoprecipitation of HA-tagged RNF113A was performed by transient expression in HEK293T cells. The cells were resuspended in high-salt buffer (50 mM Tris-HCl pH 7.9, 300 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM DTT, and protease inhibitors), lysed by sonication, and centrifuged. An equal volume of buffer containing no salt was added, and the lysate was incubated with anti-HA resin. After incubation at 4 °C with rotation, the beads were washed extensively with buffer containing 150 mM NaCl. Bound material was eluted with Laemmli buffer and analysed by SDS-PAGE. Immunoprecipitation after denaturation was performed as previously described (Sowa et al., 2009) with minor modifications. Briefly, HEK293T cells were transfected with His-Ub, then transduced with the indicated shRNA lentivirus. Cells were then treated with 500 μM MMS for 6 h and harvested. Pellets were resuspended in TBS + 1% SDS and further lysed by sonication, boiled, and cleared by centrifugation. Samples were diluted to 0.1% SDS with lysis buffer (50 mM Tris pH 7.9, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM DTT, and protease inhibitors) and incubated with Ni-NTA beads at 4 °C overnight. After incubation and extensive washing with lysis buffer, the bound material was eluted with Laemmli buffer and analyzed by western blotting.

**Structural analysis:** PyMOL (the PyMOL Molecular Graphics System, version 1.8.0.5 Schrödinger) was used to generate Figure 2.8A. ASCC2 residues 463-525 (PDB accession number 2DI0).

**shRNA library and targeted E3 ligase screen:** The targeted E3 ligase shRNA library was part of the TRC/pLKO.1 vector collection (Sigma). For the screen, U2OS cells were concurrently transduced with pHAGE-CMV-3xHA-ASCC2 lentivirus and individual lentiviral shRNAs. A scrambled pLKO.1 shRNA and a lentivirus targeting UBC13 (TRCN0000039435) served as the
negative and positive controls, respectively. After approximately 72 h, the cells were treated with MMS (500 μM) for 6 h and processed for immunofluorescence microscopy using anti-HA and pH2A.X. At least 100 cells per sample were analyzed for HA-ASCC2 foci formation. Quantified results were normalized to the scrambled control. RNF113A was the only candidate E3 ligase with three independent shRNAs exhibiting at least a threefold reduction in MMS-induced HA-ASCC2 foci formation. For verification, U2OS cells were co-transduced with pHAGE-CMV-3xHA-ASCC2 and the candidate lentiviral shRNAs. HA-ASCC2 foci formation was assessed as above.

**Statistical analyses:** All P values were calculated by unpaired, two-tailed Student’s t-test. All error bars represent the standard deviation, unless otherwise noted.
Chapter 3: RNA Ligase-Like Domain in ASCC1 Regulates ASCC Complex Function during Alkylation Damage


3.1 Abstract
Multiple DNA damage response (DDR) pathways have evolved to sense the presence of damage and recruit the proper repair factors. We recently reported a signaling pathway induced upon alkylation damage to recruit the ALKBH3-ASCC3 dealkylase-helicase repair complex (see Chapter 2). As in other DDR pathways, the recruitment of these repair factors is mediated through a ubiquitin-dependent mechanism. However, the machinery that coordinates the proper assembly of this repair complex and controls its recruitment is still poorly defined. Here, we demonstrate that the ASCC1 accessory subunit is important for the regulation of ASCC complex function. ASCC1 interacts with the ASCC complex through the ASCC3 helicase subunit. We find that ASCC1 is present at nuclear speckle foci prior to damage, but leaves the foci in response to alkylation. Strikingly, ASCC1 loss significantly increases ASCC3 foci formation during alkylation damage, yet most of these foci lack ASCC2. These results suggest that ASCC1 coordinates the proper recruitment of the ASCC complex during alkylation, a function that depends on a putative RNA-binding motif near the ASCC1 C-terminus. Consistent with its role in alkylation damage signaling and repair, ASCC1 knockout through a CRISPR/Cas9 approach results in alkylation damage sensitivity in a manner epistatic with ASCC3. Together, our results identify a critical regulator of the ALKBH3-ASCC alkylation damage signaling pathway and suggest a potential role for RNA-interacting domains in the alkylation damage response.
### 3.2 Introduction

Endogenous DNA alkylation damage is caused by numerous agents that are present in the environment, as well as by cellular metabolism (Drablos et al., 2004; Fu et al., 2012; Sedgwick et al., 2007). Exogenous alkylation damage may be induced by a number of cancer chemotherapeutics. If left unrepaired, alkylated adducts can stall replication, cause mutations, and potentially lead to cell death. Because of the diverse chemical nature of alkylation damage, multiple pathways have evolved to protect the genome from alkylation damage. These include base-excision repair (BER), direct reversal by O6-methylguanine methyltransferase (MGMT), and the AlkB family of demethylases/dealkylases (Fu et al., 2012; Sedgwick et al., 2007; Soll et al., 2017).

Although BER excises alkylated bases, it is also responsible for the removal of many other forms of DNA damage, including oxidized bases, uracil, and other deaminated bases (Krokan and Bjoras, 2013). Conversely, MGMT and the AlkB proteins appear to be dedicated solely to the direct reversal of alkylation damage (Fu et al., 2012; Sedgwick et al., 2007; Soll et al., 2017). MGMT repairs O-linked adducts by the direct transfer of an alkyl group to a cysteine in the active site via a non-enzymatic mechanism that inactivates MGMT (Xu-Welliver and Pegg, 2002; Zak et al., 1994). Conversely, AlkB proteins are genuine demethylases/dealkylases that directly reverse N-linked adducts such as 1-methyladenine (1meA) and 3-methylcytosine (3meC) in an Fe(II) and 2-oxoglutarate–dependent reaction (Falnes et al., 2002; Trewick et al., 2002). 1meA and 3meC are particularly cytotoxic as both disrupt canonical base pairing, hence blocking replicative DNA polymerases (Fu et al., 2012). In humans, there are nine AlkB homologs (Gerken et al., 2007; Kurowski et al., 2003; Sanchez-Pulido and Andrade-Navarro, 2007), but
only two of these proteins, ALKBH2 and ALKBH3, have been shown to repair 1meA and 3meC in DNA (Duncan et al., 2002).

It is important for the cell to coordinate the various alkylation repair pathways, as there is some redundancy in the substrate binding of the numerous repair factors. This overlap in substrate preference may lead to a potential conflict during initial lesion recognition and reduce the efficiency of repair. For example, alkyl-adenine glycosylase (AAG), which is involved in initiating BER, binds to the 3,N⁴-ethenocytosine (εC) lesion but cannot excise the base (Gros et al., 2004; Lingaraju et al., 2011). Interestingly, ALKBH2 is capable of repairing εC but is inhibited by the presence of AAG (Fu and Samson, 2012). Because of competition for substrates, it is important for the cell to have a tightly controlled damage response. This ensures that repair of damage lesions occurs in an efficient manner and prevents the recruitment of inappropriate repair factors. To understand the interplay between these different repair mechanisms, it is necessary to determine the regulation of the individual alkylation damage repair pathways. However, for alkylation damage repair, little is known about the regulation of repair factor recruitment in vivo.

We previously found that the ALKBH3 demethylase associates with the Activating Signal Cointegrator Complex (ASCC, also known as ASC-1) (Dango et al., 2011). These play a key role in repairing alkylated DNA in cell lines overexpressing ALKBH3, such as prostate and non-small cell lung tumor cells (Dango et al., 2011; Konishi et al., 2005; Tasaki et al., 2011). ASCC is comprised of three proteins: ASCC1, ASCC2, and ASCC3 (also known as p50, p100, and p200, respectively) (Jung et al., 2002). Biochemical characterization of this complex revealed that ASCC3 is a DNA helicase, whose unwinding activity is crucial for dealkylation by the ALKBH3 repair enzyme in vitro (Dango et al., 2011). It is thought that ASCC3 and ALKBH3
work in concert such that ASCC3 generates the single-stranded substrate needed for ALKBH3-mediated repair. Recently, we found that ASCC2 is important for the recruitment of the ALKBH3-ASCC3 complex to nuclear speckle foci specifically during alkylation damage (see Chapter 2) (Brickner et al., 2017; Galganski et al., 2017; Spector and Lamond, 2011). This recruitment depends upon nonproteasomal Lys-63-linked ubiquitination by the E3 ligase RNF113A (Brickner et al., 2017). The ubiquitination is recognized by the ASCC2 subunit, which is responsible for the recruitment of both ASCC3 and ALKBH3 to sites of damage. Loss of ASCC2 results in increased sensitivity to alkylating agents, strongly suggesting that ASCC2-mediated recruitment is critical for efficient repair (Brickner et al., 2017).

Here, we characterize ASCC1, the smallest subunit of the ASCC complex. We find that ASCC1, unlike ASCC2 or ASCC3, is constitutively present at nuclear speckle foci. ASCC1 is removed from these nuclear regions upon alkylation damage. As a result, ASCC1 is capable of modulating ASCC3 recruitment during alkylation damage. This behavior of ASCC1 depends upon its C-terminal RNA ligase-like domain. Together, our data suggest a novel regulatory mechanism for the ALKBH3-ASCC repair pathway wherein ASCC1 modulates the localization and function of the complex components.

3.3 Results
3.3.1 ASCC1 Interacts Directly with ASCC3 but is Present at Nuclear Speckle Foci in the Absence of Damage
We wished to determine which factors associated with the ASCC complex are involved in regulating its function in response to alkylation damage. To this end, we focused on ASCC1, a protein previously shown to co-purify with ASCC2 and ASCC3 (Dango et al., 2011; Jung et al., 2002). To determine how the individual complex components associate with one another, we performed immunoprecipitation of HA-ASCC1 and HA-ASCC2 (Figure 3.1A). Both of these
Figure 3.1. ASCC1 Binds Directly to ASCC3. (A) HA-tagged vector, ASCC1, or ASCC2 were expressed in 293T cells and immunoprecipitated using anti-HA resin. Immunoprecipitated (HA-IP) and input samples (1.5%) were analyzed using the indicated antibodies. The amount of ASCC3 immunoprecipitated by HA-ASCC1 was found to be 3.5% of the input, whereas HA-ASCC2 immunoprecipitated 8.4% of the input. Positions of molecular weight markers are shown on the left. (B) 293T whole-cell lysate was immunoprecipitated (IP) using anti-ASCC3 or IgG control antibodies and then Western blotted as shown. Input represents 2.5% of the IP samples. (C) GST, GST-ASCC1, or GST-ASCC2 were immobilized onto GSH-Sepharose and incubated with full-length (FL) His-tagged ASCC3 or an N-terminal deletion (NΔ-ASCC3). After washing, the bound material was analyzed by SDS-PAGE and Western blotting using anti-His antibody or by
factors co-immunoprecipitated ASCC3 from 293T cells. Consistent with this finding, immunoprecipitation of endogenous ASCC3 from 293T cell extracts yielded ASCC1, indicating that this physical interaction is present at the endogenous level (Figure 3.1B). To test whether ASCC1 and ASCC3 interact directly, we purified all three components of the complex as recombinant proteins. His-tagged ASCC3 bound to immobilized GST-tagged ASCC1, as well as GST-ASCC2, but not GST alone (Figure 3.1C). An N-terminal truncation of ASCC3 (NΔ-ASCC3; residues 401–2202) abrogated the interaction with ASCC2, but did not affect ASCC1 binding (Figure 3.1C). Thus, both ASCC1 and ASCC2 can bind directly to ASCC3, but likely through distinct regions within ASCC3. Recombinant ASCC1 and ASCC2 did not interact with one another in pulldown assays (data not shown), suggesting that ASCC3 serves as a scaffold between ASCC1 and ASCC2. To test this, we knocked out ASCC3 in PC-3 cells using CRISPR/Cas9 (Figure 3.2A). Immunoprecipitation of HA-ASCC1 from wild-type PC-3 cells yielded the other two components of the complex, but ASCC2 was not co-immunoprecipitated in the absence of ASCC3 (Figure 3.2B). Thus, ASCC3 is required to bridge the interaction between ASCC1 and ASCC2 in vivo.

As both ASCC2 and ASCC3 form nuclear foci specifically upon alkylation damage (Brickner et al., 2017), and in light of the physical interactions between the complex components, we reasoned that ASCC1 may also form alkylation-induced foci. Unlike the other components of the ALKBH3-ASCC complex, HA-tagged ASCC1 formed foci that co-localized with the nuclear speckle component PRP8 in the absence of any damage (Figure 3.3A-C). Surprisingly, treatment of the cells with the alkylating agent methyl methanesulfonate (MMS) significantly reduced ASCC1 co-localization with these nuclear domains in a time-dependent manner. This was not due to a reduction in the expression level of the tagged ASCC1 during MMS treatment (Figure
Figure 3.2. **ASCC3 is a Scaffold between ASCC1 and ASCC2.** (A) PC-3 ASCC3 knockout was generated using CRISPR/Cas9 technology. (B) HA-tagged ASCC1 was expressed in PC-3 WT or ASCC3 KO cells and immunoprecipitated using anti-HA resin. HA-IP and input samples were analyzed using the indicated antibodies.
Figure 3.3. ASCC1 is removed from Nuclear Speckles upon Alkylation Damage. (A) U2OS cells expressing HA-tagged ASCC1 were untreated or treated with MMS (0.5 mM) for 1, 2, or 6 h as shown. Cells were processed for immunofluorescence using anti-HA and anti-PRP8 antibodies, with Hoechst used as the nuclear counterstain. Scale bar, 10 μm. (B) Quantitation of (A). n = 3 biological replicates of 100 cells for each replicate, and error bars indicate ± S.D. of the mean. (C) Whole cell lysates from U2OS WT cells expressing HA-tagged ASCC1 or empty vector were analyzed by Western blotting. Whole cell lysate from U2OS WT cells expressing HA-tagged ASCC1 were untreated or treated with MMS (0.5 mM) for 6 h and analyzed by Western blotting.
Taken together, these results suggest that ASCC1 is part of the ASCC complex but may perform a distinct function in response to alkylation damage.

3.3.2 ASCC1 Modulates Alkylation-Induced ASCC3 Foci Formation
We next wished to determine the role of ASCC1 in ASCC3 foci formation. To this end, we knocked out ASCC1 in U2OS cells using CRISPR/Cas9 (Figure 3.4A-B). Notably, loss of ASCC1 significantly increased MMS-induced ASCC3 foci formation (Figure 3.4C-D). This increase was apparent with two different knockout clones, making it unlikely that the induction of ASCC3 foci was due to an off-target effect of CRISPR/Cas9. These results were not attributable to an increase of ASCC3 foci at baseline (i.e. without MMS) in the ASCC1 KO cells (Figure 3.5). In time-course experiments, ASCC3 foci were still resolved in the absence of ASCC1 upon removal of MMS (Figure 3.5). These results suggested that ASCC1 modulates ASCC3 foci formation during alkylation damage.

We next asked whether ASCC1 influences the established co-localization of the other components of the ASCC complex. Upon MMS treatment, nearly 75% of WT cells had co-localizing ASCC3 and HA-ASCC2 foci (Figure 3.6A-B). Under the same conditions, ASCC1 KO cells had significantly fewer cells with co-localizing HA-ASCC2 and ASCC3 foci (42%). This was not due to a difference in the expression level of HA-ASCC2 in parental versus ASCC1 KO cells (Figure 3.6C). This suggested that ASCC1 may function to promote the association of the other two components of the complex during alkylation damage. To test this biochemically, we immunoprecipitated endogenous ASCC2 in WT versus ASCC1 knockout PC-3 cells (Figure 3.6D). Consistent with the diminished interaction observed by microscopy, less ASCC3 was co-immunoprecipitated with ASCC2 in ASCC1 knockout cells than in WT cells upon alkylation.
Figure 3.4. Loss of ASCC1 Results in Increased ASCC3 Foci Formation. (A) U2OS ASCC1 knockout was generated using CRISPR/Cas9 technology. (B) Whole cell lysate from WT and two U2OS ASCC1 KO clones were analyzed by Western blotting. (C) U2OS WT and ASCC1 KO cells were treated with MMS (0.5 mM) for 6 h and processed for immunofluorescence using anti-ASCC3 and anti-pH2A.X antibodies, with Hoechst as the nuclear counterstain. (D) Quantification of (C). n = 3 biological replicates of 100 cells per replicate, and error bars indicate ± S.D. of the mean. * = p < 0.01.
Figure 3.5. **Time Course of ASCC3 Foci Formation.** Quantification of ASCC3 foci in WT and ASCC1 KO cells treated with MM (0.5 mM) for 6 h at indicated recovery times. n = 3 biological replicates of 100 cells per replicate, and error bars indicate ± S.D. of the mean.
Figure 3.6. ASCC1 Modulates the Co-Localization of ASCC2 and ASCC3 during Alkylation Damage. (A) U2OS WT and ASCC1 KO cells expressing HA-tagged ASCC2 were treated with MMS (0.5 mM) for 6 h. Cells were processed for immunofluorescence using anti-ASCC3 and anti-HA antibodies, with Hoechst as the nuclear counterstain. (B) Quantification of (A). Only cells with ≥ 5 ASCC3 foci were scored. n = 3 biological replicates of 100 cells per replicate, and error bars indicate ± S.D. of the mean. * = p < 0.01. (C) Whole cell lysates from U2OS WT and ASCC1 KO cells expressing HA-tagged ASCC2 were analyzed by Western blotting. (D) Whole cell lysate from WT and two PC-3 ASCC1 KO clones were analyzed by Western blotting. (E) PC-3 WT and ASCC1 KO cells were treated with MMS (0.5 mM) for 6 h. Whole cell lysate was immunoprecipitated using anti-ASCC2 or IgG control antibodies and then Western blotted as shown. Inputs represent 2.5% of the IP samples.
damage (Figure 3.6E). Thus, ASCC1 appears to coordinate the proper recruitment of the complex components during alkylation.

3.3.3 Deletion Analysis of ASCC1 Reveals Modular Functional Domains

We reasoned that distinct domains within ASCC1 may be responsible for the interaction with ASCC3 and its removal from the nuclear speckle domains during damage. ASCC1 contains a KH domain adjacent to an unstructured region at its N-terminus, as well as an RNA ligase-like C-terminus, which has been postulated to be an RNA-binding domain (Brown et al., 2003; Siomi et al., 1993; Valverde et al., 2008). We created deletion mutants of ASCC1 (Figure 3.7A) and tested their ability to associate with ASCC3. Deletion of the N-terminus of ASCC1 (ASCC1-NΔ; residues 54–357) abolished binding to ASCC3, whereas deletion of the C-terminus (ASCC1-CΔ; residues 1–243) had no effect on this interaction (Figure 3.7B).

We then expressed the ASCC1-NΔ and ASCC1-CΔ constructs in ASCC1 knockout cells (Figure 3.4A-B) to prevent possible interference from endogenous ASCC1. We analyzed the ability of the ASCC1 mutants to retain localization within nuclear speckles upon MMS treatment. Strikingly, HA-ASCC1-CΔ maintained foci formation, whereas HA-ASCC1-NΔ behaved like WT ASCC1 (Figure 3.8A-B). This was not because ASCC1-CΔ was expressed at a higher level than WT ASCC1 or ASCC1-NΔ (Figure 3.8C). Thus, modular domains within ASCC1 have distinct functions during the alkylation damage response.

3.3.4 Putative RNA-Binding Domain in ASCC1 Regulates ASCC Function

In analyzing the C-terminal RNA ligase-like domain of ASCC1, we noticed that it contains two conserved His-X-Thr motifs (HXT), which have been shown to be important for RNA or nucleotide binding in various proteins (Koonin and Gorbunov, 1990). Examples of other proteins containing this motif in their nucleotide-binding pocket include the 2′–5′ RNA ligases
Figure 3.7. ASCC1 N-Terminal Domain Interacts with ASCC3. (A) Schematic of human ASCC1 domain structure and mutants (to scale). (B) HA-tagged ASCC1 FL or indicated ASCC1 deletions were expressed in 293T cells and immunoprecipitated using anti-HA resin. HA-IP and input samples were analyzed using the indicated antibodies.
Figure 3.8. Deletion Analysis of ASCC1 Reveals Modular Functional Domains. (A) U2OS ASCC1 KO cells expressing HA-tagged ASCC1 or indicated ASCC deletions were treated with MMS (0.5 mM) for 6 h. Cells were processed for immunofluorescence using anti-HA and anti-PRP8 antibodies, with Hoechst used as the nuclear counter stain. (B) Quantification of (A). n = 3 biological replicates of 100 cells per replicate, and error bars indicate ± S.D. of the mean. (C) Whole cell lysates from U2OS cells expressing HA-tagged ASCC1 or indicated ASCC1 deletions were collected and analyzed by Western blotting as shown.
from *Thermus thermophilus* and *Pyrococcus horikoshii*, as well as the AMP-binding protein AKAP18 (also known as AKAP7) (Figure 3.9A) (Koonin and Gorbalenya, 1990; Mazumder et al., 2002). Previous structural studies suggest that these HXT motifs line the substrate-binding pocket and interact with the nucleotide through a pseudo 2-fold symmetry (Gold et al., 2008). We modeled this domain within ASCC1 using the Phyre2 server (Kelley et al., 2015; Kelley and Sternberg, 2009). The resulting structural analysis suggested that ASCC1 forms a similar overall structure to other members of the 2H phosphoesterase family (Figure 3.9B) (Silverman and Weiss, 2014). Furthermore, the predicted structure suggests that the conserved HXT motifs of ASCC1 are positioned such that they also line a putative nucleotide- or RNA-binding pocket similar to the aforementioned RNA ligases and AKAP18. Notably, the ASCC1 domain lacks residues critical for ligase activity (Doherty and Suh, 2000). We then mutated both of the HXT motifs of ASCC1 to AXA (ASCC1-AXA: 179HLT → 179ALA and 277HAT → 277AAA) and analyzed its localization during MMS damage. As with ASCC1-CΔ, ASCC1-AXA retained foci under these conditions (Figure 3.10). This indicates that the HXT motifs of ASCC1 play a role in its localization during alkylation damage.

Because the C-terminus of ASCC1 appeared to be critical for regulating its ability to form foci, we asked whether the RNA ligase-like domain also impacted the foci formation of the other complex components during alkylation damage. To address this question, we rescued ASCC1 knockout cells by expressing exogenous ASCC1 WT, ASCC1-CΔ, or ASCC1-AXA (Figure 3.11A). Although the WT ASCC1 partially rescued HA-ASCC2/ASCC3 foci co-localization, neither ASCC1-CΔ nor ASCC1-AXA was able to rescue this phenotype (Figure 3.11B-C). Thus, the putative RNA ligase-like domain of ASCC1 plays an important role in the regulation of ASCC complex localization upon alkylation damage.
**Figure 3.9. ASCC1 Contains a Putative RNA-Binding Domain.** (A) Schematic of the human ASCC1 protein is shown on top, with the positions of the HXT motifs (to scale). Sequence alignment with human AKAP18 is shown on bottom. The dual HXT motifs are highlighted in green, and neighboring conserved residues are highlighted in blue. (B) Predicted structure of ASCC1 (residues 132–355). The HXT motifs are indicated by arrows. This domain was modeled using the Phyre2 server.
Figure 3.10. See next page for caption.
Figure 3.10. HXT Motifs Play a Role in ASCC1 Localization during Alkylation Damage. (A) U2OS WT and ASCC1 KO cells expressing HA-tagged ASCC1 or indicated ASCC deletions were treated with MMS (0.5mM) for 6 h. Cells were processed for immunofluorescence using anti-HA and anti-PRP8 antibodies, with Hoechst used as the nuclear counterstain. (B) Quantification of (A). Only cells with $\geq 5$ ASCC3 foci were scored. n=3 biological replicates of 100 cells, and error bars indicate ± S.D. of the mean. (C) U2OS ASCC1 KO cells expressing HA-tagged ASCC1 or indicated ASCC1 mutant were untreated or treated with MMS (0.5 mM) for 6 h as indicated. Cells were processed for immunofluorescence using anti-HA and anti-PRP8 antibodies, with Hoechst as the nuclear counterstain. (D) Quantification of (C). n = 3 biological replicates of 100 cells per replicate, and error bars indicate ± S.D. of the mean. (E) Whole cell lysates from U2OS WT and ASCC1 KO cells expressing HA-tagged ASCC1 FL or indicated ASCC1 mutant were
Figure 3.11. ASCC1 RNA Ligase-Like Domain Regulates ASCC2 and ASCC3 Foci Formation. (A) Whole cell lysates from U2OS WT and ASCC1 KO cells expressing HA-tagged ASCC2 and untagged ASCC1 FL or indicated ASCC1 mutant were analyzed by Western blotting. Asterisk (*) indicates Ig heavy chain. (B) U2OS WT and ASCC1 KO cells expressing HA-tagged ASCC2 and untagged ASCC1 WT or indicated mutations were treated with MMS (0.5 mM) for 6 h. Cells were processed for immunofluorescence using anti-HA and anti-ASCC3 antibodies, with Hoechst used as the nuclear counterstain. (C) Quantification of (B). n = 6 biological replicates of 100 cells, and error bars indicate ± S.D. of the mean. *= p < 0.001; # = p < 0.05; n.s., not significant.
3.3.5 Role of ASCC1 in Alkylation Damage Resistance
The previous results suggest that ASCC1 may play a key role in modulating ASCC recruitment during alkylation damage. We then tested whether or not ASCC1 is functionally important for alkylation damage resistance in PC-3 prostate cancer cells. ASCC1 was knocked out in these cells using CRISPR/Cas9 (Figure 3.6D). Loss of ASCC1 resulted in an increase in sensitivity to MMS as compared to the WT cells (Figure 3.12A). Again, the increase in sensitivity was observed with two distinct ASCC1 knockout clones. To determine whether this decrease in cell survival in response to MMS was due to the function of ASCC1 within the ASCC complex, or whether this was due to its function in another pathway, we created ASCC1/ASCC3 double knockout cells (ASCC1/3 DKO). We sequentially knocked out ASCC3 and then ASCC1 in PC-3 cells using CRISPR/Cas9 (Figures 3.2A and 3.12B-C). MMS sensitivity of all four resulting genotypes was then tested. Consistent with our previous work, loss of ASCC3 increased sensitivity to MMS (Dango et al., 2011). However, the ASCC1/3 DKO cells did not have an additional increase in MMS sensitivity as compared with either the ASCC1 KO or the ASCC3 KO cells (Figures 3.12D). These results support the notion that ASCC1 has an epistatic relationship with ASCC3 in alkylation damage resistance. Taken together, our data support a role for ASCC1 in controlling the ASCC complex recruitment and function during the cellular response to alkylation damage.

3.4 Discussion
We recently described a signaling pathway that is activated by alkylation damage to recruit the ALKBH3-ASCC complex to nuclear foci (Brickner et al., 2017). This pathway depends upon the RNF113A E3 ligase, which induces Lys-63–linked ubiquitination that is then recognized by the ASCC2 subunit (Brickner et al., 2017). Here, we present evidence for additional regulation of this pathway by ASCC1. Our work suggests that ASCC1 is constitutively present at nuclear
Figure 3.12. ASCC1 KO Cells are Sensitive to Alkylation Damage. (A) PC-3 ASCC1 KO cells were assessed for sensitivity to MMS relative to WT PC-3 cells. Cell survival was measured by MTS assay. n = 5, and error bars indicate ± S.D. of the mean. (B) Strategy for creating PC-3 ASCC1/3 double-knockout (DKO) cells using CRISPR/Cas9 technology. Deep sequencing results are shown at the bottom. (C) Whole cell lysates from PC-3 cells, as well as the PC-3 ASCC1/3 DKO cells, were collected and analyzed by Western blotting as shown. (D) PC-3 ASCC1 KO, ASCC3 KO, and ASCC1/3 DKO cells were assessed for sensitivity to MMS relative to WT PC-3 cells. Cell survival was measured by MTS assay. n = 5, and error bars indicate ± S.D. of the mean.
speckle foci prior to damage, but leaves these foci upon MMS treatment. In addition, ASCC1 can interact directly with ASCC3 and thus modulate its localization during alkylation damage. Consistent with a role in this pathway, knockout of ASCC1 sensitizes cells to alkylation damage. Loss of ASCC1 does not further increase the sensitivity of cells that lack ASCC3, suggesting that the role of ASCC1 in the alkylation damage response is primarily through the ASCC complex.

Surprisingly, unlike ASCC2 or ASCC3, ASCC1 is already present at nuclear foci in the absence of any damage. Upon alkylation damage, ASCC1 is removed from these foci (Figure 3.3A-B). This phenomenon depends on the C-terminal domain of ASCC1 and, more specifically, its HXT motifs (Figures 3.8 and 3.10). At the same time, ASCC1 can bind directly to ASCC3 via its N-terminus (Figure 3.1). This physical interaction and the dynamic localization of ASCC1 during alkylation is the basis for our preferred model to explain the resulting phenotypes from ASCC1-deficient cells (Figure 3.13). We hypothesize that ASCC1 is acting as a specificity determinant for ASCC3 localization at these foci. In WT cells, we observe that the vast majority of the ASCC3 foci are positive for ASCC2 (Figure 3.6). In the ASCC1 knockout cells, ASCC3 foci are significantly increased, yet the majority of these lack ASCC2. Thus, there are likely two subsets of ASCC3 foci; those that are positive for ASCC2 and those that are negative for ASCC2. In WT cells, the fraction of ASCC3 present at foci without ASCC2 is likely removed in a manner dependent on ASCC1. This is consistent with our observation that more ASCC3 foci form in ASCC1 knockout cells. The failure of ASCC3 to be removed from these ASCC2-negative foci by ASCC1 would explain why we observe more ASCC3 foci that lack ASCC2 in the ASCC1 knockout cells. Our immunoprecipitation results further confirm this notion (Figure 3.6E).

Why does an increase in ASCC3 foci formation lead to increased alkylation damage sensitivity? This phenotype is potentially due to the necessary regulation of ASCC3 recruitment by ASCC1.
Figure 3.13. Proposed Model for ASCC Complex Localization during Alkylation Damage. In WT cells, RNF113A-mediated ubiquitination is recognized by ASCC2 and recruits ASCC3 to nuclear speckle foci. Simultaneously, a fraction of ASCC3 is recruited to ASCC2-negative foci, but these are removed by ASCC1. This activity of ASCC1 depends upon the C-terminal RNA ligase-like domain via the engagement of an unknown ligand (X). In ASCC1 KO/C-terminal mutant cells (bottom), the fraction of ASCC3 foci that are independent of ASCC2 is significantly increased. See text for details.
In double-stranded break repair, the loss of the repair protein 53BP1 increases BRCA1 recruitment, but this leads to increased sensitivity to γ-irradiation, at least partly due to the recruitment of BRCA1 in the G1 phase of the cell cycle (Daley and Sung, 2014). This inappropriate recruitment and attempt at homologous recombination in G1 is thought to be deleterious in double-strand break repair. In a like manner, in the absence of ASCC1, inappropriate ASCC3 recruitment may cause alkylation damage sensitivity because other repair factors are displaced, or a portion of ASCC3 needs to be removed for repair to be promptly completed. It is also possible that, in the absence of ASCC1, the complex cannot function properly and alkylation damage sensitivity is increased despite an increase in ASCC3 recruitment.

The C-terminal RNA ligase-like domain of ASCC1, which appears to be critical for the function described here, is part of a larger 2H phosphoesterase family of enzymes that have been shown to harbor diverse activities, including bona fide tRNA ligases, phosphodiesterases, and putative RNA-binding factors (Mazumder et al., 2002; Silverman and Weiss, 2014). Structural studies on the phosphoesterase domain of AKAP18 initially suggested a proclivity for binding to AMP and CMP in a manner that depends upon its HXT motifs (Gold et al., 2008). It is intriguing that AKAP18 binds to the same nucleotides that are the major reaction products for the ALKBH3 dealkylase activity, which primarily targets 1-methyladenine and 3-methylcytosine for demethylation. We currently do not have any direct evidence for the binding of ASCC1 to AMP or CMP. However, the importance of this domain in ASCC1 foci formation strongly implies that substrate binding through this domain, whatever its biochemical identity, plays a role in ASCC complex recruitment and function.
3.5 Materials and Methods

**Plasmids:** Human ASCC1 cDNA was isolated by RT-PCR from total human RNA, cloned into pENTR-3C (Invitrogen), and subcloned into pMSCV-FLAG, pMSCV-FLAG-HA, or pHAGE-CMV-HA3 by Gateway recombination (Sowa et al., 2009). ASCC1 deletions and point mutations were created by PCR and cloned as above. ASCC2, ASCC3, and ALKBH3 vectors were previously described (Brickner et al., 2017). For recombinant protein expression, WT ASCC1, ASCC1 mutants, and ASCC2 were subcloned into pGEX-4T1 or pET28a-FLAG. For expressing the His6-tagged full-length ASCC3 and NΔ-ASCC3 (401–2202), the pENTR-3C vectors containing these cDNAs were subcloned into pDEST10 (Invitrogen). All constructs produced by PCR were verified by Sanger sequencing.

**CRISPR/Cas9-mediated knockouts:** U2OS and PC-3 KO cells were created using CRISPR/Cas9 genome editing at the Genome Engineering and iPSC Center (GEiC) at Washington University School of Medicine (St. Louis). The U2OS ASCC3 KO cells were previously described (Brickner et al., 2017). The gRNA sequences used to generate the ASCC1 KO cell lines were 5′-AAGGATTCGCTCTACTTTGNGG-3′ and 5′-AAGTAGACCGGAATCCTTGTNGG-3′. The gRNA sequence used to generate the PC-3 ASCC3 KO cell line was 5′-GACATTTGAAAAGGAACGCANGG-3′. All knockout clones were verified by deep sequencing or by Western blot analysis.

**Cell culture, viral transduction, and cell survival assays:** U2OS, PC-3, 293T, and Sf9 cells were cultured and maintained as described previously (Zhao et al., 2015). Preparation of viruses, transfection, and viral transduction were also performed as described previously (Zhao et al., 2015). For knockout cell foci rescue experiments, U2OS cells were transduced with WT ASCC1 or ASCC1 mutants using the pMSCV retroviral vector and pHAGE-CMV-HA3-ASCC2. For
DNA damaging agent survival assays using PC-3 cells, 4000–15,000 cells/well were cultured overnight in 96-well plates in 100 μl of media. Cells were then exposed to medium containing the indicated concentration of MMS (Sigma) for 24 h at 37 °C. The media were then replaced with normal media, and cell viability was assessed 72 h after initial exposure to MMS via the MTS assay (Promega). All MTS-based survival experiments were carried out in quintuplicate.

**Recombinant protein purification:** For purification of the His6-tagged ASCC3 and NΔ-ASCC3, the baculovirus vector was produced using the Bac-to-Bac expression system (ThermoFisher Scientific). Amplified baculovirus was used to infect Sf9 cells and harvested after 72 h. The cells were resuspended in Buffer L (20 mm Tris, pH 7.3, 150 mm NaCl, 8% glycerol, 0.2% Nonidet P-40, 0.1% Triton X-100, 20 mm imidazole) plus protease inhibitors and frozen −80 °C. Cells were lysed by sonication and rotated for 30 min at 4 °C. The cell extracts were then centrifuged at 10,000 rpm for 10 min. The supernatant was incubated with nickel-nitrilotriacetic acid beads and eluted with Buffer L containing 400 mm imidazole. His-ASCC1 and GST-tagged recombinant proteins were purified from E. coli as described (Zhao et al., 2015). All proteins were dialyzed into TAP buffer (50 mm Tris, pH 7.9, 100 mm KCl, 5 mm MgCl2, 0.2 mm EDTA, 0.1% Nonidet P-40, 10% glycerol, 2 mm 2-mercaptoethanol, 0.2 mm phenylmethylsulfonyl fluoride) after purification.

**Protein binding assays:** All *in vitro* GST-protein binding assays were performed as described previously (Mosammaparast et al., 2013) with minor modifications. Briefly, 5 μg of GST-tagged bait protein was incubated with 10 μl of GSH-Sepharose beads and 250 ng of His6 ASCC3 FL or NΔ-ASCC3, 1 μg of His6 ASCC1, or 500 ng of Lys-63–Ub(3–7) in TAP buffer containing 1% BSA in a total volume of 100 μl. After incubation at 4 °C with rotation for 1 h, beads were
washed extensively using TAP buffer. Bound material was eluted using Laemmli buffer and analyzed by SDS-PAGE and Western blotting.

**Structural model:** The model for the predicted structure of ASCC1 was generated using the publicly available Phyre2 server (Kelley et al., 2015; Kelley and Sternberg, 2009).

**Statistical analysis:** All p values were calculated by the unpaired two-tailed Student's t test.

**Immunofluorescence microscopy:** All immunofluorescence microscopy was done as described previously (Brickner et al., 2017; Mosammaparast et al., 2013). 100 cells were analyzed at least in biological triplicate for all quantifications.

**Immunoprecipitation and Western blotting:** Immunoprecipitation of HA-tagged ASCC1, ASCC1 mutants, and ASCC2 was performed by transfection of constructs into 293T cells using Transit293 reagent (Mirus Bio). Cells were treated with 0.5 mm MMS as indicated, collected, washed in 1× PBS, and frozen at −80 °C. Cell pellets were resuspended in IP lysis buffer (50 mm Tris, pH 7.9, 300 mm NaCl, 10% glycerol, 1% Triton X-100, 1 mm DTT, and protease inhibitors), lysed by sonication, and cleared by centrifugation. An equal volume of IP lysis buffer containing no salt was added (final concentration of NaCl was 150 mm). Lysates were then incubated with anti-HA resin (Santa Cruz Biotechnology) for 3-4 h at 4 °C with rotation. The beads were washed extensively with IP lysis buffer containing 150 mm NaCl, and bound material was eluted with Laemmli buffer.

Preparation of viruses, transfection, and viral transduction for immunoprecipitation of HA-tagged ASCC1 or HA-empty from PC-3 WT and ASCC3 KO cells was performed as described previously (Mosammaparast et al., 2013). Cells were selected with 1 μg/ml puromycin for 24 h. The media were then replaced with normal media for 2 days, after which cells were transfected
with FLAG-ASCC2. Cells were collected and washed in 1× PBS and frozen at −80 °C 2 days after transfection. Immunoprecipitation was then executed as described above.

Endogenous immunoprecipitation was carried out by collecting the cells and freezing at −80 °C as above. Cell pellets were resuspended in TAP buffer containing 300 mm KCl, lysed by sonication, and cleared by centrifugation. IP lysis buffer containing no salt was added to bring the final concentration of KCl to 100 mm. Samples were pre-cleared by incubation with protein A/G beads (Santa Cruz Biotechnology) with rotation at 4 °C. After centrifugation, the supernatant was then incubated with the relevant antibodies overnight at 4 °C. Protein A/G beads were then added and rotated at 4 °C for 1 h. The samples were then centrifuged and washed extensively with TAP buffer. Bound material was eluted with Laemmli buffer and analyzed by Western blotting.
Chapter 4: Conclusion and Future Directions

The ALKBH2 and ALKBH3 dealkylases are overexpressed in certain tumors, such as prostate adenocarcinoma and non-small cell lung carcinoma (Konishi et al., 2005; Tasaki et al., 2011).

This is of particular interest because alkylating agents are clinically used in the treatment of various tumors and both ALKBH2 and ALKBH3 are able to repair 1meA and 3meC lesions (Duncan et al., 2002). ALKBH3 has been shown to interact with the ASCC complex. The ASCC complex is comprised of the DNA helicase, ASCC3, as well as two other proteins, ASCC1 and ASCC2, which had not been previously defined.

The research presented here provides insight into how these accessory proteins regulate ALKBH3-ASCC recruitment during alkylation damage, and therefore, ALKBH3 dealkylation activity in cells. We find that ASCC1 and ASCC2 are critical for complex assembly and recruitment, respectively. This knowledge is critical in order to have a comprehensive understanding of DNA alkylation damage repair in vivo.

4.1 ASCC2 Ubiquitin-Dependent Signaling in Alkylation Damage Repair

The enzymatic mechanism of repair by the ALKBH3 dealkylase has been well-established (Duncan et al., 2002; Falnes et al., 2002; Trewick et al., 2002). However, little was known about the recruitment of ALKBH3 to 1meA and 3meC damage adducts, the preferred substrate of ALKBH3. Here, our lab establishes a critical role for ASCC2 in the recruitment of the ALKBH3-ASCC complex to sites of alkylation damage.

ALKBH3, ASCC2 and ASCC3 all form nuclear foci after treatment with alkylating agents (Figures 2.1 and 2.2). These foci not only co-localize with one another but also with the alkylated lesion 1meA, indicating that these factors are recruited to sites of damage (Figure 2.3).
Furthermore, immunofluorescence and mass spectrometry experiments both substantiate the notion that ALKBH-ASCC co-localizes with BRR2, PRP8, and elongating RNA polymerase II (Figure 2.4). These are all factors that are important for active transcription and splicing. This suggests that the ALKBH-ASCC complex may have an important role in the repair of alkyl lesion near actively transcribed genes and could even be involved in splicing.

The recruitment of ASCC3 and ALKBH3 to foci is dependent upon ASCC2. Analysis of the ASCC2 protein sequence revealed a highly conserved CUE domain (residues 467-509), which belongs to the ubiquitin-binding domain superfamily (Komander and Rape, 2012). We found that recombinant ASCC2 bound to K63-linked ubiquitin chains in vitro (Figure 2.7B). ASCC2 and K63-Ub also co-localized in vivo upon alkylation damage (Figure 2.7C). Introducing ASCC2 point mutations predicted to be critical for the recognition of ubiquitin abrogated ASCC2-ubiquitin binding in vitro (Figure 2.8B). Experiments with WT and CUE domain ASCC2 mutants revealed that the ASCC2 CUE domain mutants that abrogated ubiquitin binding in vitro had diminished foci formation as compared to the WT ASCC2 protein (Figure 2.8C). Thus, the recruitment ASCC2 to sites of alkylation damage is dependent upon K63-linked ubiquitin binding.

ASCC2 ubiquitin binding not only had an effect on ASCC2 foci formation, it also had an effect on both ASCC3 and ALKBH3 foci formation. ASCC2 KO cells have significantly reduced ASCC3 and ALKBH3 foci formation (Figure 2.9). This reduction in ASCC3 and ALKBH3 foci was only rescued by the re-expression of ASCC2 WT protein but not an ASCC2 ubiquitin-binding mutant (Figure 2.11). Thus, ASCC2 ubiquitin binding is important for the recruitment of ASCC2, ASCC3, and ALKBH3 to sites of alkylation damage which establishes ubiquitin signaling as important for the repair of alkyl lesions by ALKBH3.
We next looked at the physical interactions between ASCC2, ASCC3, and ALKBH3. Through a combination of in vitro binding assays with recombinant protein and in vivo co-immunoprecipitation assays, we determined that ASCC3 binds to both ASCC2 and ALKBH3 through distinct regions of the ASCC3 protein (Figure 2.13). ALKBH3 and ASCC2 do not interact with one another. This supports the conclusion that ASCC2 bridges the interaction of K63-linked ubiquitin with ASCC3 and that ALKBH3 is recruited through a tertiary interaction with ASCC3 (Figure 2.13E).

RNF113A was found to be the E3 ubiquitin ligase responsible for the upstream ubiquitin signaling that results in the recruitment of the ALKBH3-ASCC complex during alkylation damage. We identified RNF113A through a screen using a custom library of shRNAs that target UBC13-interacting E3 ligases or other E3 ligases implicated in DNA repair. Expressing shRNAs targeting RNF113A in cells resulted in reduced alkylation induced HA-ASCC2 foci formation (Figure 2.14A). Through a mass spectrometry based ASCC2 interactome analysis, we were able to identify BRR2 as one substrate for RNF113A that impacted foci formation of complex components (Figures 2.15 and 2.17). Knockdown of BRR2 resulted in a significant reduction of ASCC3 foci formation upon alkylation damage. This indicates a role for BRR2 in this alkylating damage specific signaling pathway.

Together, this data identifies a function of ASCC2 in promoting repair by the ALKBH3-ASCC complex. ASCC2 serves as a critical pathway component for the recruitment of ALKBH3 and ASCC3 to sites of alkylation damage. Furthermore, the spliceosomal component BRR2 was found to be upstream of ASCC2 in the signaling cascade, providing evidence that the ALKBH3-ASCC complex may intersect with splicing in vivo.
4.1.1 Future Directions

Additional future studies could focus on the impact that ALKBH3-ASCC dependent alkylation damage repair has on splicing. ALKBH3 is able to repair single-stranded RNA as well as single-stranded DNA. In light of the association of ASCC2 with the aforementioned spliceosomal and mRNA processing factors and the ability of ASCC3 to bind RNA (Figure 2.5A), I hypothesize that the ASCC complex is important for the repair of alkylated RNA. To test whether or not the ASCC complex is necessary for the repair of RNA in vivo, global levels of alkylated 1meA and 3meC RNA will be determined by mass spectrometry. The levels of methylated RNA can be quantified for MMS treated PC-3 control, ASCC2 KO, and ALKBH3 KO cell lines by first separating the RNA and DNA from the cell lysates by TRIzol extraction (Rio et al., 2010). The RNA will then be digested with an S1 nuclease (Sigma) and FastAP Alkaline Phosphatase (ThermoFisher Scientific). The resulting samples can then be analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to determine the quantity of alkylated RNA. (Su et al., 2014; Thuring et al., 2016). I predict that there will be more 1meA and 3meC RNA lesions in the ASCC2 and ALKBH3 knockouts as compared to the control cells. This experiment could be rescued by transfecing wild type or mutant protein into the aforementioned knockout cells.

It is currently unclear what influence alkylated RNA has on RNA processing. It is possible that damaged RNA inhibits or obstructs the splicing process. Consequently, the ALKBH3-ASCC complex may not only be critical for the reversal of RNA damage, but also for splicing. To assess splicing efficiency in response to alkylation damage, an in vivo double-reporter splicing assay can be used (Nasim and Eperon, 2006). This assay is performed by comparing the signal of the upstream β-galactosidase reporter to the downstream luciferase reporter. The downstream reporter is only expressed if splicing has occurred. Splicing efficiency can be determined by
measuring the ratio of the reporters from the spliced and unspliced RNA transcripts. Splicing can be assessed for PC-3 wild type, ASCC2 KO, and ALKBH3 KO cells with and without MMS treatment after transfection with the reporter plasmid. These experiments will establish the role of the ALKBH3-ASCC complex in promoting pre-mRNA splicing.

4.2 Regulation of the ALBH3-ASCC Complex by ASCC1 Ligase-Like Domain

Our previous work established that ALBH3, ASCC2, and ASCC3 all form foci in response to alkylation damage (Brickner et al., 2017; Dango et al., 2011). Unlike the other components of the ALKBH3-ASCC complex, ASCC1 forms foci that co-localize with the splicing component PRP8 in the absence of damage. Surprisingly, treatment with the alkylating agent MMS results in a significant reduction in ASCC1 foci (Figure 3.3A-B). This indicates that ASCC1 plays a distinct role from the other complex components in response to alkylation damage.

ASCC1 and ASCC3 interact directly (Figure 3.1). Due to this interaction, we wanted to determine if depleting ASCC1 would have an effect on the formation of alkylation induced ASCC3 foci. Unexpectedly, there was an increase in ASCC3 foci in ASCC1 KO cells, as compared to the WT control cells, after treatment with MMS (Figure 3.4C-D). This suggests that a function of ASCC1 may be to negatively regulate ASCC3 foci formation. We next asked whether ASCC1 affects the co-localization of the other ASCC complex components. Upon MMS treatment, the majority of WT cells had co-localizing ASCC3 and HA-ASCC2 foci. This co-localization was significantly reduced in ASCC1 KO cells (Figure 3.6). Therefore, while ASCC2 and ASCC3 can interact directly, their co-localization and recruitment to foci appears to be dependent upon ASCC1 in vivo.
We next determined which domains within ASCC1 were responsible for its removal from nuclear speckle domains during alkylation damage. ASCC1 contains two putative RNA-binding domains, a KH domain and a C-terminal RNA ligase-like domain. Included in the latter domain are two conserved His-X-Thr motifs (Mazumder et al., 2002) found in members of the 2H phosphoesterase family (Silverman and Weiss, 2014). These motifs have been shown to be important for nucleotide binding in other proteins. ASCC1-NΔ, ASCC1-CΔ, and ASCC1-AxA (His-X-Thr motif mutant) were expressed in ASCC1 KO cells. Foci formation was analyzed after MMS treatment. Strikingly, HA-ASCC1-CΔ and ASCC1-AxA retained foci after treatment with MMS, while HA-ASCC1-NΔ behaved like WT ASCC1 (Figures 3.8 and 3.10). Therefore, the putative RNA ligase-like domain of ASCC1 plays an important role in regulating ASCC1 localization upon alkylation damage. Consistent with the aforementioned immunofluorescence findings, ASCC1 WT, but neither ASCC1-CΔ nor ASCC1-AxA, was able to partially rescue HA-ASCC2 and ASCC3 co-localization (Figure 3.11).

ASCC2, ASCC3, and ALKBH3 all promote resistance to alkylation damage as assessed by MTS assay (Brickner et al., 2017; Dango et al., 2011). We determined that ASCC1 is also important for alkylation damage resistance using this assay. PC-3 knockout cells deleted of ASCC1, ASCC3, or ASCC1/3 were assessed for cell survival by the MTS assay. ASCC1 deficient cells were hypersensitive to the alkylating agent MMS in a manner that was epistatic with ASCC3 (Figure 3.12).

Together, this data supports the following model; we hypothesize that ASCC1 is acting as a specificity determinant for ASCC3 localization at foci and as such is regulating this complex during alkylation. In WT cells, we observed that the vast majority of the ASCC3 foci are positive for ASCC2. In the ASCC1 knockout cells, ASCC3 foci are significantly increased, yet the
majority of these foci lack ASCC2. Thus, there are likely two subsets of ASCC3 foci: those that are positive for ASCC2 and those that are negative for ASCC2. In WT cells, RNF113A mediated ubiquitination is recognized by ASCC2 and recruits ASCC3 to nuclear speckle foci. Simultaneously, a fraction of ASCC3 is recruited to ASCC2 negative foci by an independent mechanism, but these are removed by ASCC1. This activity of ASCC1 is dependent upon the C-terminal RNA ligase-like domain via the engagement of an unknown ligand. Thus, in ASCC1 KO/C-terminal mutant cells, the fraction of ASCC3 foci that are independent of ASCC2 is significantly increased (Figure 3.13).

4.2.1 Future Directions
There is still much to be discovered about the activity of ASCC1. Future research should focus on determining the structure and potential enzymatic activity of ASCC1. We are working together with collaborators in the laboratory of John Tainer to solve the structure of the RNA ligase-like domain of ASCC1. Their group has solved an X-ray crystal structure of this domain to 2.8Å resolution (unpublished, Roopa Thapar). The structure indicates that the His-X-Thr motifs are positioned in a manner similar to the 2H phosphodiesterase (Song et al., 2010) family member AKAP18 (Figure 3.9B). AKAP18 is a phosphodiesterase capable of degrading 2′-5′ oligoadenylate (OA) (Gold et al., 2008; Gusho et al., 2014). Due to the sequence and structural similarities between ASCC1 and AKAP18, I hypothesize that ASCC1 is a PDE.

The ligand for ASCC1 has not yet been determined. Due to the predicted RNA binding motifs of ASCC1, it is most likely that RNA is the substrate for ASCC1 activity. Additionally, the surface of the ASCC1 ligase-like domain is highly negatively charged (unpublished observation, Roopa Thapar). I predict that ASCC1 will preferentially bind to methylated RNA substrates. This would be consistent with a role for ASCC1 in the alkylation damage repair response. Specifically,
1meA and 3meC are the most likely candidate substrates for ASCC as these ALKBH3 substrates are both positively charged adducts (Dominissini et al., 2016; Koivisto et al., 2004; Roundtree et al., 2017).

RNA immunoprecipitation coupled with mass spectrometry (RIP-MS) could be used to determine the ligand for ASCC1. For this experiment, Flag-tagged ASCC1 WT and RNA ligase-like domain mutant ASCC1 AxA would be overexpressed in cells. The ASCC1 AxA mutant will be used as a negative control for ligand binding as these conserved residues are critical for RNA or nucleotide binding in numerous proteins (Koonin and Gorbalenya, 1990). The work shown here, that ASCC1 AxA alters ASCC1 foci formation during alkylation damage, indicates that these residues are important for the function of ASCC1 (Figure 3.10). Following immunoprecipitation of the tagged protein, RNA will be isolated by TRIzol extraction (Rio et al., 2010). The resulting RNA will then be digested with an S1 nuclease (Sigma) and FastAP Alkaline Phosphatase (ThermoFisher Scientific). The resulting samples can then be analyzed by mass spectrometry to determine the preferred ASCC1-RNA interaction. Once the substrate is identified, it would be interesting to attempt to solve the crystal structure of ASCC1 in complex with the ligand.

If ASCC1 is a PDE, then the activity may be affected by the presence of alkylation damage. The implication of this hypothesis is that the degradation of RNA by ASCC1 is a signal that regulates an alkylation-specific damage response. To test this in vitro, recombinant ASCC1 WT or ASCC1 AxA could be incubated with short single-stranded RNA substrates. Degradation of the RNA substrate by ASCC1 would be assessed by simple electrophoresis. If ASCC1 is an RNase, it would be of import to determine if there is a change in the reaction kinetics with a methylated substrate. Furthermore, the interaction between ASCC1 and ASCC3 could activate or inhibit
ASCC1 activity. This could be assessed by the addition of recombinant ASCC3 into the aforementioned assay.

Finally, performing RNAseq in WT and ASCC1 KO cells with and without MMS induced alkylation damage could provide insight into changes in the global transcriptome. The results generated from a RNAseq experiment would give information not only about changes in gene expression before and after alkylation but also about alternative splicing (Kukurba and Montgomery, 2015). With this data, we would have a better understanding of how ASCC1 impacts the overall cellular response to alkylation damage extending beyond the ALKBH3-ASCC repair pathway.


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