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DNA unwinding by ASCC3 helicase is coupled to ALKBH3 dependent DNA alkylation repair and cancer cell proliferation

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Summary

Demethylation by the AlkB dioxygenases represents an important mechanism for repair of N-alkylated nucleotides. However, little is known about their functions in mammalian cells. We report the purification of the ALKBH3 complex and demonstrate its association with the Activating Signal Co-integrator Complex (ASCC). ALKBH3 is overexpressed in various cancers, and both ALKBH3 and ASCC are important for alkylation damage resistance in these tumor cell lines. ASCC3, the largest subunit of ASCC, encodes a 3′-5′ DNA helicase, whose activity is crucial for the generation of single-stranded DNA upon which ALKBH3 preferentially functions for dealkylation. In cell lines that are dependent on ALKBH3 and ASCC3 for alkylation damage resistance, loss of ALKBH3 or ASCC3 leads to increased 3-methylcytosine and reduced cell proliferation, which correlates with pH2A.X and 53BP1 foci formation. Our data provide a molecular mechanism by which ALKBH3 collaborates with ASCC to maintain genomic integrity in a cell type specific manner.

Introduction

The genome is continuously subjected to various harmful insults, such as ionizing radiation and nucleic acid modifying compounds. Alkylation is one of the mechanisms by which nucleic acids can be altered and its continuous repair is important to genomic integrity. Alkylating agents are ubiquitous in the environment, but a major source of alkylation damage is thought to be due to normal cellular metabolism (Ringvoll et al., 2006; Rydberg and Lindahl, 1982; Sedgwick, 2004). In mammalian cells, several possible sources of non-

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enzymatic methylation by methyl group donors exist, but only the universal methyl donor *S*-adenosylmethionine has been shown to non-enzymatically methylate DNA (Barrows and Magee, 1982). In fact, the estimated endogenous DNA methylation corresponds to an exposure of cells to 20 nM of methyl methanesulfonate (MMS), a potent alkylating agent (Rydberg and Lindahl, 1982).

A number of mechanisms exist to repair alkylation damage, including base excision repair (BER), direct reversal by methylguanine methyltransferase (MGMT), and dealkylation repair via the AlkB family of enzymes (Sedgwick, 2004). Originally discovered in *E. coli*, AlkB belongs to a large family of non-heme Fe(II) and 2-oxoglutarate-dependent dioxygenases, which catalyze a host of important biological reactions, such as proline hydroxylation and histone demethylation (Mosammaparast and Shi, 2010; Sundheim et al., 2008). Bacterial AlkB demethylates alkylated bases such as 1-methyladenine (1meA) and 3-methylcytosine (3meC) by oxidation of the N-linked methyl moiety. This creates an unstable methyl-iminium intermediate that is spontaneously hydrolyzed to form formaldehyde and a non-alkylated base (Falnes et al., 2002; Trewick et al., 2002). In *E. coli*, absence of AlkB leads to a moderate hypersensitivity to MMS and a significantly increased rate of MMS-mediated base substitution mutations (Delaney and Essigmann, 2004). Mammalian cells have at least eight AlkB family members, ALKBH1 through ALKBH8 (Drablos et al., 2004). Only two of the corresponding human proteins, ALKBH2 and ALKBH3, have been convincingly shown to possess repair activity similar to the *E. coli* AlkB protein *in vitro* (Aas et al., 2003; Duncan et al., 2002; Lee et al., 2005). Biochemical experiments suggest that ALKBH2 prefers double-stranded DNA (dsDNA) substrates, while ALKBH3 prefers single-stranded DNA (ssDNA) substrates and also demethylates 1meA and 3meC in RNA (Aas et al., 2003). Mouse knockout studies have demonstrated that the loss of *AlkBh2* causes accumulation of 1meA in genomic DNA as well as enhanced MMS sensitivity (Ringvoll et al., 2006). In the same study, these phenotypes were not observed in the *AlkBh3* knockout mice (Ringvoll et al., 2006). These results, as well as the preference of ALKBH3 for single-stranded substrates, have raised the question as to whether ALKBH3 has any role in maintaining genomic integrity (Falnes et al., 2004; Ringvoll et al., 2006), although redundancy amongst AlkB family members remains a possibility. Furthermore, despite extensive *in vitro* characterization of these enzymes, how they access alkylated DNA substrates and whether they play a general or lineage-specific role under physiological or pathological conditions remain unanswered.

Here, we investigate the biological function and molecular mechanism of action of ALKBH3, initiated by purification of ALKBH3 and its associated partner proteins from human cells. We found that ALKBH3 associated with an uncharacterized complex, the Activating Signal Co-integrator Complex (ASCC). Interestingly, ALKBH3 has recently been shown to be overexpressed in a subset of cancers, including prostate and non-small cell lung carcinoma (Konishi et al., 2005; Tasaki et al., 2011). We demonstrate that in tumor cell lines that overexpress ALKBH3, both ALKBH3 and ASCC3, the largest subunit of ASCC, are important for alkylation damage resistance. Importantly, ASCC3 encodes a 3′-5′ DNA helicase, an activity which is present in the ALKBH3 complex and co-purifies with ASCC3. We show that ASCC3 unwinds DNA to generate the single-stranded substrate needed for ALKBH3-mediated DNA repair, thus revealing a molecular mechanism that involves coupling of a helicase and a dealkylating enzyme in the maintenance of genomic integrity. Strikingly, we found that loss of ALKBH3 or ASCC3 significantly reduces cell proliferation, both in culture and in a xenograft model, of tumor cell lines dependent on this pathway for alkylation damage resistance. This correlated with the accumulation of endogenous 3meC in genomic DNA, as well as formation of pH2A.X and 53BP1 foci, suggesting activation of the DNA damage response. Our findings uncover a DNA repair enzyme complex with a potential role in specific tumor types, and highlight a molecular

mechanism by which a dealkylating enzyme repairs damaged DNA to maintain genomic integrity.

Results

Biochemical characterization of the ALKBH3 complex

To gain greater insight into the *in vivo* function of ALKBH3, we purified the ALKBH3 complex from nuclear extracts of 293T cells using the sequential double-affinity tag purification technique described previously (Nakatani and Ogryzko, 2003). The resulting ALKBH3 complex (Figure 1a) was subjected to tandem mass spectrometry. Besides ALKBH3 itself, we identified numerous peptides corresponding to three subunits of the Activating Signal Co-integrator Complex (ASCC; Figure 1a and Supplemental Table 1a). The three subunits, ASCC1, ASCC2, and ASCC3 (also named p50, p100, and p300, respectively), were previously found to be associated with a transcriptional activation complex (Jung et al., 2002). However, the precise molecular function of this complex is thus far unclear. The largest subunit, ASCC3, is predicted to encode a superfamily II-type helicase, although to our knowledge, no enzymatic activity has been yet described. We also purified the ALKBH3 complex from HeLa cells and found all three components of the ASCC complex (data not shown).

Since previous work had demonstrated that ALKBH3 is overexpressed in prostate cancer (Konishi et al., 2005), we wished to determine whether the ALKBH3-ASCC association was relevant in this pathophysiological context. To this end, we also purified the ALKBH3 complex from PC-3 cells, an androgen-independent prostate cancer cell line in which ALKBH3 is relatively overexpressed (see below). All three components of the ASCC complex were present (Figure 1b). Western blotting readily confirmed the presence of ASCC components in the ALKBH3 complex purified from PC-3 cells (Figure 1b, bottom panel). To further investigate this association, we carried out glycerol gradient sedimentation analysis of the PC-3 ALKBH3 complex and analyzed the protein fractions by silver staining, immunoblotting, and mass spectrometry (Figure 1c and Supplemental Table 1b). Most of the ALKBH3 was found to be present at the top of the gradient (fractions 3-9), likely representing the uncomplexed form of the protein. A small but significant portion of ALKBH3 was also present in fractions 11-17. Importantly, Western blotting (Figure 1c, middle panel) and mass spectrometry (Supplemental Table 1b) confirmed the presence of ASCC3 primarily in these fractions. To further confirm the ALKBH3-ASCC3 interaction, we carried out reciprocal co-immunoprecipitation and showed that ALKBH3 can be immunoprecipitated by an ASCC3 antibody from the Flag-purified ALKBH3 complex (Figure 1d), as well as from endogenous whole-cell extracts (Figure 1e). Together, these findings strongly suggested that ALKBH3 interacts with the ASCC complex *in vivo*.

Loss of ALKBH3 and ASCC3 causes alkylation damage sensitivity in specific cancer cell lines

Because ALKBH3 has been reported to be overexpressed in prostate cancer and non-small cell lung cancer (Konishi et al., 2005; Tasaki et al., 2011), we determined the expression of ALKBH3 in various cancer cell lines in search of an appropriate model system to study ALKBH3 and ASCC. Western blotting of whole cell extracts confirmed that ALKBH3 is relatively overexpressed in both PC-3 and LNCaP prostate cancer cell lines, but not in the non-malignant prostate epithelial cell line RWPE-1 (Figure 2a), although ALKBH3 is expressed in RWPE-1 cells (see Supplemental Figure S1b). We also found that ALKBH3 is relatively overexpressed in H23 cells, a non-small cell lung adenocarcinoma cell line, but not in WiDr (colon adenocarcinoma), U2OS (osteosarcoma), or HTB-1 (urothelial carcinoma) cells (Figure 2a). Interestingly, a similar pattern of ASCC3 expression was

observed in these same cell lines (Supplemental Figure S1a). We postulated that in cells that overexpress ALKBH3, ALKBH3 may play an important role in alkylation damage resistance. The fact that ASCC associates with ALKBH3 suggested that the former may also play a role in this process. To address these possibilities, ALKBH3 or ASCC3 were stably knocked down using lentiviral shRNA constructs in a host of cell lines (Supplemental Figure S1b). These cells were then exposed to increasing concentrations of the alkylating agent MMS, and survival was assessed using a tetrazolium based colorimetric survival assay (MTS; (Ringvoll et al., 2006)). Strikingly, we found that in both PC-3 as well as LNCaP cells, knockdown of either ALKBH3 or ASCC3 resulted in a significantly increased MMS sensitivity compared to the control knockdown (Figure 2b-d). This phenotype was also observed in the H23 lung carcinoma cell line (Figure 2e). Importantly, knockdown of ALKBH2, another AlkB homologue, did not result in increased alkylation sensitivity in PC-3 cells (Supplemental Figure S2), suggesting a specific role for ALKBH3 in these cells.

A previous genetic study demonstrated that *AlkBh3* does not play a significant role in alkylation damage resistance in mouse embryonic fibroblasts (Ringvoll et al., 2006). We therefore asked whether ALKBH3 or ASCC3 are important for alkylation damage resistance in other human cell lines that do not overexpress ALKBH3. We failed to detect significantly increased alkylation sensitivity in the non-malignant prostate epithelial cell line RWPE-1 (Figure 2f), consistent with the previous study. We also failed to detect this phenotype in HTB-1, WiDr, or U2OS cell lines (Figure 2g-i). Taken together, these results uncover an important role for ALKBH3 and its partner ASCC3 in the repair of alkylation damage in a cell line specific manner, probably reflecting the ALKBH3 expression profile.

ALKBH3 catalytic activity is important for alkylation damage resistance

Is the catalytic activity of ALKBH3 important for its role in alkylation damage resistance observed in prostate cancer cells? To address this issue, we stably expressed tagged, shRNA-resistant wildtype or catalytically inactive ALKBH3 (H257A; (Lee et al., 2005)) in PC-3 cells, with concurrent knockdown of the endogenous ALKBH3 (Figure 3a). Exposure of these cells to increasing concentrations of MMS demonstrated a rescue of the alkylation hypersensitivity phenotype with the wildtype but not the catalytically inactive form of ALKBH3 (Figure 3b). Our results suggested that MMS sensitivity of PC-3 cells is dependent on the ALKBH3 catalytic activity.

ASCC3 is a 3′-5′ DNA helicase

Previous work suggested that relative to ALKBH2, ALKBH3 has significantly less dealkylation activity when confronted with a double-stranded DNA substrate relative to single-stranded substrates (Aas et al., 2003). Thus, it was surprising that ALKBH3, but not ALKBH2, appeared to play an important role in alkylation damage resistance in the above cell lines. We hypothesized that ALKBH3 may function in dsDNA dealkylation repair because of its association with ASCC3, which encodes a putative helicase. To characterize the potential helicase activity of ASCC3, we first attempted but failed to express and purify full-length, recombinant ASCC3 in insect cells. We therefore subcloned three fragments of ASCC3 covering the entire open reading frame of ASCC3 (termed ASCC3-A, ASCC3-B, and ASCC3-C), which consisted of amino acid residues 1-400, 401-1300, and 1301-2202, respectively (Figure 4a). The ASCC3-B and ASCC3-C domains both share homology with the yeast Mer3 protein, which has been shown to be important for DNA damage repair as a Holliday junction helicase (Nakagawa and Kolodner, 2002). The three fragments of ASCC3 were purified as GST fusion proteins (Supplemental Figure S3) and were tested for their ability to interact with ALKBH3 and their DNA helicase activity *in vitro*. Both GST-ASCC3-B and GST-ASCC3-C, but not GST-ASCC3-A, were able to interact with recombinant ALKBH3, suggesting a direct interaction between these proteins (Figure 4b).

Using previously described ^{32}P-labeled DNA substrates ((Nakagawa and Kolodner, 2002); Supplemental Table 2), helicase activity was tested using all three recombinant fragments and a number of different DNA substrates. As shown in Figure 4c, the C-terminal ASCC3-C fragment was capable of unwinding DNA with a 3′-(lane 9), but not a 5′-overhang (lane 5), a Holliday junction (lane 13), or a blunt-ended substrate (data not shown). Importantly, the Flag-purified ALKBH3 complex displayed the same substrate specificity as the recombinant ASCC3 protein (Figure 4c, lanes 6, 10 and 14 and data not shown), suggesting that native ASCC3 also functions in the 3′-5′ direction. In contrast, fragments A and B of ASCC3 (ASCC3-A or ASCC3-B) had no activity towards any of the substrates (Figure 4c, lanes 3, 4, 7, 8, 11 and 12, and data not shown). Previous work on yeast Mer3 showed that a glycine residue, which is part of its ATP-binding domain, is critical for its activity; when mutated to aspartate, Mer3 helicase activity is lost (Nakagawa and Kolodner, 2002). The analogous glycine was similarly mutated in ASCC3-C (G1354D), which was subsequently purified as a GST-fusion (Supplemental Figure S3). Indeed, this mutation also abrogated the 3′-5′ DNA helicase activity of recombinant ASCC3 (Figure 4d).

Because the ALKBH3 complex had helicase activity similar to recombinant ASCC3, and MS/MS analysis of the ALKBH3 complex did not reveal any other putative helicases, we reasoned that ASCC3 was likely to be responsible for the observed activity of the ALKBH3 complex. To provide further evidence that this was the case, we subjected the purified ALKBH3 complex fractions (see Figure 1c) to the helicase assay using the 3′-overhang DNA substrate. Only fractions 11-13 contained detectable helicase activity (Figure 4e), which coincided with the peak ASCC3 fractions. Taken together, our results strongly suggested that ASCC3 is a DNA helicase with 3′-5′ directionality.

ASCC3 promotes ALKBH3-mediated repair of an alkylated adduct on dsDNA *in vitro*

Since ASCC3 can unwind dsDNA, and ALKBH3 has been previously shown to prefer single-stranded substrates for dealkylation, we hypothesized that ASCC3 promotes dsDNA dealkylation repair by generating the appropriate substrate for ALKBH3. To test this hypothesis, we first confirmed the preference of recombinant ALKBH3 to repair 3-methylcytosine (3meC) on ssDNA relative to dsDNA. 3meC demethylation on ssDNA versus dsDNA was assessed using an oligonucleotide containing this lesion in the context of a HpaII restriction site (Supplemental Table 3) using the scheme described in Figure 5a. Increasing amounts of ALKBH3 efficiently repaired 3meC on ssDNA, but not on dsDNA (Figure 5b, compare lanes 4-6 and 7-9). We then tested the ability of the Flag-purified ALKBH3 complex to demethylate 3meC on dsDNA. Indeed, the ALKBH3 complex was able to repair this lesion *in vitro* while the recombinant ALKBH3 had no detectable activity (Figure 5c, compare lane 4 with lanes 6-7), suggesting that additional factor(s) are present in the complex which facilitated this repair. Importantly, addition of wildtype, recombinant ASCC3-C, but not the catalytically inactive mutant (G1354D) was sufficient to promote dsDNA repair mediated by recombinant ALKBH3 (Figure 5d, compare lanes 6-7 with 9-10.). In the absence ALKBH3, recombinant ASCC3-C did not possess any detectable 3meC repair activity (Supplemental Figure S4). Thus, although ALKBH3 prefers ssDNA as a substrate, the presence of its helicase partner ASCC3 enables it to repair alkylated dsDNA.

ALKBH3 and ASCC3 are required for proliferation in a cell-dependent manner

Do ALKBH3 and ASCC3 have a functional role in the absence of exogenous alkylation damage? We hypothesized that these repair systems are likely present to protect the genome from the effects of endogenous alkylation damage. Since alkylation damage can inhibit cell proliferation through a variety of mechanisms (Drablos et al., 2004), we asked whether either protein is important for proliferation in the cell lines that overexpress ALKBH3. Strikingly, using the MTS assay, we observed a significantly decreased rate of proliferation

with ALKBH3 or ASCC3 knockdown in the same cells that required these proteins for alkylation resistance (Figure 6a-c and Supplemental Figure S5), suggesting that ALKBH3 and ASCC3 both promote proliferation of these cancer cells even in the absence of MMS. Proliferation was not affected in the cell lines which do not overexpress ALKBH3 (Figure 6d-f and Supplemental Figure S5). We further confirmed these findings in PC-3 cells by analyzing total cell counts (Figure 6g), which again demonstrated a similarly reduced rate of cell proliferation (*K*, defined as the doubling rate per day, was as follows: $K_{shGFP} = 0.466$; $K_{shALKBH3} = 0.285$; and $K_{shASCC3} = 0.244$; $p = 0.004$ and $p = 0.011$, respectively).

To further demonstrate the importance of the ALKBH3-ASCC3 complex in prostate tumor cell proliferation, we knocked down ALKBH3 and ASCC3 in PC-3 cells, and xenograft tumor growth was examined in castrated male SCID mice relative to control knockdown. As shown in Figure 6h, knockdown of ALKBH3 resulted in significantly reduced tumor formation in the xenograft model, relative to control knockdown. Knockdown of ASCC3 demonstrated a similar phenotype (Figure 6i). Representative mice demonstrating these phenotypes as well as their respective excised tumors are shown in Figure 6j and k. Taken together, our data demonstrate that ALKBH3 and its associated partner ASCC3 are important for cell proliferation in these established cancer cell lines.

ALKBH3 and ASCC3 prevent accumulation of 3meC in specific cancer cells

To determine whether ALKBH3 and ASCC3 promote 3meC repair in cells, we developed an antibody that specifically recognizes 3meC. As shown in Supplemental Figure S6, the antibody detected an oligonucleotide carrying 3meC (row B), but showed no cross-reactivity with unmodified, 5-methylcytosine (5meC) or 5-hydroxymethylcytosine (5hmC) containing oligonucleotides (row A). Furthermore, the antibody also recognized both double- and single-stranded genomic DNA (Supplemental Figure S6, rows C and D). To determine whether ALKBH3 and ASCC3 regulate 3meC levels in the cell, we knocked down either ALKBH3 or ASCC3 in PC-3 cells and then measured the global genomic 3meC levels using the 3meC-specific antibody. As shown in Figure 7a, knockdown of either ALKBH3 or ASCC3 resulted in a significant increase in the 3meC level in these cells (compare rows B and C with A) but had no impact on 5meC. Significantly, knockdown of ALKBH3 or ASCC3 had no significant impact on the overall level of 3meC in the RWPE-1 non-malignant prostate epithelial cell line (Figure 7a). These data suggest that both ALKBH3 and ASCC3 are important for 3meC repair in specific cancer cell lines, even in the absence of exogenous alkylation damage. These results also correlate with the cell-specific loss in proliferation discussed earlier, and suggest that the increased 3meC levels in genomic DNA may be the cause for this phenotype.

Loss of ALKBH3 and ASCC3 activates the pH2A.X DNA damage signaling pathway

Accumulation of alkylation damage inhibits DNA replication fork progression, leading to double-strand breaks, and activation of the DNA damage response pathway, which is initiated by phosphorylation of the variant histone H2A.X (pH2A.X; also known as γH2A.X; (Nikolova et al., 2010)). We hypothesized that if endogenous alkylation damage accumulated in the absence of ALKBH3 and ASCC3 in a cell type specific manner, it was possible that this damage-signalling pathway could be activated. Indeed, knockdown of ALKBH3 or ASCC3 resulted in the phosphorylation of H2A.X in PC-3 cells, but not in RWPE-1 cells, in the absence of exogenous DNA damage (Figure 7b). To confirm our findings, we performed immunofluorescent microscopy on both cell lines and analyzed formation of pH2A.X DNA damage foci after knockdown of ALKBH3 or ASCC3. Knockdown of either protein resulted in the formation of pH2A.X foci in many of the PC-3, but not RWPE-1 cells (Figure 7c). These foci co-localized with those of 53BP1, consistent with activation of this pathway. These results suggested that in the absence of ALKBH3 or

ASCC3, DNA damage signalling is initiated in PC-3 cells, which is likely due to the accumulation of 3meC or similar lesions. Taken together, our data indicate that ALKBH3 and its associated helicase partner ASCC3 are important for DNA alkylation repair and genomic maintenance in a cell specific manner.

Discussion

The *E. coli* AlkB enzyme was identified nearly ten years ago as an Fe^{+2}-dependent dioxygenase that catalyzes the oxidative demethylation of certain alkylated lesions, particularly 1-methyladenine and 3-methylcytosine (Falnes et al., 2002; Trewick et al., 2002). Soon thereafter, two mammalian homologues of AlkB, ALKBH2 and ALKBH3, were shown to have similar activities (Aas et al., 2003; Duncan et al., 2002; Lee et al., 2005). One significant difference between the two mammalian enzymes was that while ALKBH2 catalyzed repair on double-stranded DNA, ALKBH3 preferred single-stranded substrates *in vitro* (Aas et al., 2003; Lee et al., 2005). Thus, the role of ALKBH3 was thought to be primarily in repair of alkylated RNA (Falnes et al., 2004). Recent work also suggested that ALKBH3 is overexpressed in various cancers, specifically in prostate cancer and non-small cell lung cancer (Konishi et al., 2005; Tasaki et al., 2011), but whether it plays a role in genomic stability has been questioned due to its substrate preference. We provide evidence that ALKBH3 mediates repair of alkylated, double-stranded DNA in concert with an uncharacterized helicase, further elucidating the molecular mechanism by which ALKBH3 functions and demonstrating its role in promoting genomic stability in a cell specific manner.

The requirement for ALKBH3 for alkylation resistance in certain cancer cell lines appears to be highly specific. For instance, in prostate and lung cancer cell lines, knockdown of ALKBH3 resulted in hypersensitivity to MMS (Figure 2). On the other hand, knockdown of ALKBH3 had no significant effect on MMS resistance in a non-malignant prostate epithelial cell line or a number of other cancer cell lines tested (Figure 2). The basis for this selectivity may be at least partially explained by the relative overexpression of ALKBH3 in those responding cell lines. What leads to ALKBH3 overexpression or dependence on this pathway is not entirely clear. Notably, cell lines that depended on the ALKBH3 pathway appeared to be independent of ALKBH2 for alkylation resistance (Supplemental Figure S2), which may suggest a reciprocal nature of these two enzyme pathways. It is likely that repair of 3-methylcytosine and other similar alkylation adducts in most cells is mediated by other members of the Abh family, such as ALKBH2, or by alternate mechanisms that are yet to be identified. Indeed, recent studies using knockout mice demonstrated that ALKBH2 is the primary oxidative demethylase that repairs these lesions in non-malignant tissues (Ringvoll et al., 2008; Ringvoll et al., 2006).

Mechanistically, consistent with these previous findings, we found a cell specific increase in the 3meC levels in response to ALKBH3 knockdown (Figure 7), which suggested that ALKBH3 was required for repairing such DNA lesions in certain contexts. Repair of endogenous alkylation damage via ALKBH3 was critical in these cell lines, since knockdown of ALKBH3 led to a significant reduction in cell proliferation, both in culture and in a xenograft mouse model (Figure 6). Alkylated DNA bases can be strong inhibitors of DNA replication if left unrepaired, which may lead to DNA breaks. In these cells, we observed the activation of the double-stranded DNA damage signalling pathway upon ALKBH3 knockdown (Figure 7). This correlated with the reduced cell proliferation, which suggested that the accumulation of such damage was likely responsible for the observed phenotypes. Taken together, these results strongly suggested that ALKBH3 plays a role in maintaining genomic stability, and thus its role is not limited simply to RNA demethylation, as was suggested previously (Falnes et al., 2004; Ringvoll et al., 2006).

Recent studies have explored the structural mechanism responsible for the ssDNA preference of ALKBH3 relative to ALKBH2 (Chen et al., 2010; Monsen et al., 2010). While both ALKBH2 and ALKBH3 have similar overall structures, a divergent β-hairpin near the active site of both enzymes is responsible for substrate selectivity. Swapping this domain in ALKBH3 with the β-hairpin of ALKBH2 resulted in dealkylation activity of dsDNA substrates (Chen et al., 2010; Monsen et al., 2010). These structural differences may explain the need of ALKBH3 for a DNA helicase to perform its function on genomic DNA. Our proteomics and biochemical investigations identified the ASCC complex as a partner for ALKBH3. We showed that ASCC3 can unwind double-stranded DNA *in vitro* via a 3′-5′ helicase activity (Figure 4), generating single-stranded DNA, upon which ALKBH3 may act. This association expands the repair substrate repertoire of ALKBH3 to include double-stranded DNA, which we demonstrate using the purified mammalian ALKBH3 complex, as well as reconstituted, recombinant ALKBH3 and ASCC3 (Figure 5). Importantly, a point mutation in the ATPase domain of ASCC3 rendered it incapable of contributing to ALKBH3-mediated repair of double-stranded DNA. Thus, the biochemical association between ALKBH3 and ASCC3 underscores the functional relationship between these two proteins. Consistently, knockdown of ASCC3 also led to a reduced proliferation and increased MMS sensitivity in the same cells, which are dependent on ALKBH3, similar to what we observed for the ALKBH3 knockdown cells.

The physical and functional association between these two proteins highlights a molecular mechanism by which ALKBH3 repairs alkylated DNA in concert with ASCC (Figure 7). To date, the function of the ASCC complex is relatively unexplored. The three subunits of the ASCC complex were initially identified as proteins associated with human ASC-1, a transcriptional co-activator (Jung et al., 2002). Our findings that ASCC3 functions with ALKBH3 underscore the notion that the cell shares machinery to access the chromatin substrate for a variety of functions, whether it is for DNA repair or for transcription. This coupling is reminiscent of other examples where transcription and DNA repair intersect, such as the involvement of TFIIH in nucleotide excision repair (Lehmann, 2003). It is tempting to speculate that ALKBH3 may preferentially function in regions of the genome that are enriched for ASCC, making its repair activity non-random genome-wide.

3meC is produced by cellular metabolism but can also be induced by exogenous alkylating agents (Sedgwick et al., 2007). In the past, identification and quantitation of such DNA adducts was difficult due to a lack of appropriate reagents and the scarcity of 3meC relative to most other methylation marks (Sedgwick, 2004). We developed a very specific and highly sensitive antibody against 3meC, which allowed us to assess global genomic 3meC levels. Importantly, this antibody does not recognize 5meC, even at relatively high concentrations (Supplemental Figure 6). It is interesting to note that different cell types have different levels of 3meC at steady state (Supplemental Figure 6). It is possible that one of the determinants of the dependency on the ALKBH3/ASCC pathway may be the degree of endogenous 3meC, or other similar lesions, such as 1meA, although evidence suggests that ALKBH3 may repair 3meC more efficiently than 1meA (Aas et al., 2003). Using this unique reagent, we have demonstrated that loss of ALKBH3 or ASCC3 resulted in the accumulation of 3meC in a prostate cancer cell line but not in other cells (Figure 7). Consistent with this, our genetic complementation experiments demonstrated that the catalytic activity of ALKBH3 is critical for the MMS sensitivity phenotype. Taken together, these findings raise the exciting possibility that ALKBH3 may be a viable target for therapeutic intervention in pathological contexts where it is overexpressed, such as prostate cancer.

Experimental Procedures

Plasmids

Human ALKBH2, ALKBH3, ASCC1, and ASCC3 cDNAs were isolated by RT-PCR and cloned initially into pENTR-3C or pENTR/D-Topo (Invitrogen). For TAP-tag purification, ALKBH3 was subcloned into pMSCV-Flag-HA (Sowa et al., 2009). For recombinant protein production, full-length ALKBH3 was cloned into pET-28a and ASCC3 fragments were cloned into pGEX-4T1. For ALKBH2 and ALKBH3 antigen production, coding sequences corresponding to residues 1-100 of each were subcloned into pET-28a. For ASCC1 and ASCC3 antigen production, coding sequences corresponding to residues 1-150 and 1-400, respectively, were cloned into pET-28a. Mutants in ALKBH3 and ASCC3 were produced by PCR-mediated site-directed mutagenesis. For the shRNA-resistant ALKBH3 clone, six silent mutations were introduced in the in the ORF region corresponding to the sequence targeted by shALKBH3 #1 and subcloned into pMSCV-Flag-HA containing a blasticidin resistance gene. All clones were verified by sequencing. Lentiviral shRNA constructs for ALKBH2, ALKBH3, and ASCC3 were obtained from Open Biosystems/ Thermo Scientific (clones used were as follows: shALKBH3 #1: TRCN0000064748; shALKBH3 #2: TRCN0000064752; shALKBH2 #1: TRCN0000064678; shALKBH2 #2 TRCN0000064680; shASCC3 #1: TRCN0000052186; and shASCC3 #2: TRCN0000052187).

TAP-tag purification of ALKBH3 complex and MS/MS identification

The detailed purification procedure has been described previously (Nakatani and Ogryzko, 2003). Briefly, Flag-HA-tagged ALKBH3 was expressed after transduction of pMSCV-Flag-HA-ALKBH3 retrovirus into 293T or PC-3 cells. Nuclear extract was prepared from the established stable cell lines, and the ALKBH3 complex was purified using anti-Flag (M2) mAb-conjugated agarose beads (Sigma), followed by anti-HA (F-7) mAb-conjugated agarose beads (Santa Cruz) in TAP buffer (50 mM Tris-HCl pH 7.9, 100 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 0.1% NP-40, 1mM DTT, and protease inhibitors). The resulting ALKBH3-associated complex (ALKBH3.com) components were identified by LC-MS/MS of trypsin digested and zip-tipped samples using an LTQ-XL mass spectrometer (Thermo) as previously described (Sowa et al., 2009). Peptides were assigned using Sequest with a 2% peptide FDR. For glycerol gradient ultracentrifugation, an 11-40% glycerol gradient was prepared as previously described (Shi et al., 2003). The Flag-purified complex was centrifuged at 55,000 rpm in a SW55Ti rotor for 10 hours, and 200μl fractions were subsequently collected manually and analyzed.

Purification of recombinant proteins and GST-binding assays

GST- and His-tagged proteins were overexpressed in *E. coli* and purified using glutathione-Sepharose or Ni-NTA agarose, respectively, as previously described (Mosammaparast et al., 2002). All recombinant proteins were dialyzed into TAP buffer after purification. GST-binding assays were performed as previously described (Mosammaparast et al., 2002), except that TAP buffer was used for washing.

Antibodies

Rabbit anti-ALKBH2 and anti-ALKBH3 antibodies were produced by Millipore (Catalog #07-1567, and #09-882, respectively) using the His-tagged antigens described above. Rabbit anti-ASCC1 and anti-ASCC3 antibodies were produced by Open Biosystems/Thermo Scientific using the His-tagged antigens described above. Other antibodies used were: anti-HA (Covance) and anti-Flag (Sigma) as described previously (Mulligan et al., 2008; Shi et

al., 2003). Rabbit anti-3-methylcytosine (3MeC) antibody was produced in rabbit as a hapten conjugate (Active Motif).

DNA cytotoxicity assessment and proliferation assays

MMS sensitivity and cell proliferation assays were performed essentially as described (Ringvoll et al., 2006). Briefly, 4,000 cells or 2,000 cells were plated for MMS sensitivity assays and proliferation assays, respectively, in 96-well containers in 100μl media. For assessment of MMS sensitivity, cells seeded for 24 hours were exposed to medium containing an increasing concentration (0-5 mM) of methyl methanesulphonate (MMS) in culture medium for one hour at 37°C. MMS containing media was then replaced with normal media and cell growth was assessed using the MTS assay (Promega) 48 hours or 72 hours after MMS exposure. Cell proliferation was assessed using the MTS assay for 4 days starting 24 hours after cell plating. All experiments were carried out at least in triplicate.

In Vivo Tumor growth in Xenograft Models

PC-3 cells were infected with shRNA-expressing lentivirus as described above. After selection for 48-72 hours, cells were maintained in DMEM until 80-90% confluence was reached. After trypsinization and collection, cell number was adjusted to $2x10^5$ cells in 100μl ice-cold PBS, then injected into the flank of the mice. Tumor growth assays were carried out in NOD.Cg-Prkdc^{scid}IL2rg^{tm1Wjl}/SzJ male mice strains (Jackson Laboratory, stock #005557) as described previously (Craft et al., 1999). All procedures involving animals were approved by the Harvard Center for Comparative Medicine (HCCM) (Protocol #04749) at Harvard Medical School. Tumor cells were allowed to grow in 5 castrated male mice for each group for 4-10 weeks until the tumor reached a volume of ~400mm^3. Tumor volume was calculated by the formula $0.5236 \times r^2_1 \times r_2$ (where $r_1 < r_2$) (Long et al., 2000).

DNA Helicase assays

Preparation of DNA substrates was carried out as described previously (Nakagawa and Kolodner, 2002). Briefly, oligonucleotide T70 (see Supplemental Table 2 for all sequences) was 5′-end labelled with [γ-^{32}P]ATP (PerkinElmer Life Sciences) and T4 polynucleotide kinase (New England Biolabs). Oligonucleotides used to create the Holliday junction, blunt, 3′-, and 5′-overhang substrates were annealed to ^{32}P-labeled T70 in a 2:1 molar ratio in annealing buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 500 mM NaCl). Helicase assays were performed using 1 nM annealed substrate in helicase buffer (20 mM Tris-HCl pH 7.6, 10 mM NaCl, 1.5 mM $MgCl_2$, 100 μg/ml BSA, 2 mM DTT, 2 mM ATP) in a total volume of 20 μl containing the indicated amount of protein or ALKBH3 complex. Reactions were incubated at 37°C for 30 minutes, then stopped by addition of 5 μl of stop buffer (50 mM Tris-HCl pH 7.6, 50 mM EDTA, 2.5% SDS) with 0.5 μl of 25 mg/ml proteinase K and incubated for an additional 10 minutes at 37°C. Reaction products were analyzed by non-denaturing PAGE using TBE buffer; the gels were dried and visualized using a Phosphorimager.

DNA repair reactions

The sequence of the 3-methylcytosine (3MeC) containing oligonucleotide (Midland Certified Reagent Company) is shown in Supplemental Table 3. The oligonucleotide was ^{32}P-labelled as described above, and repair reactions were carried out with ALKBH3 complex or recombinant proteins in buffer containing 20 mM Tris pH 7.9, 40 mM KCl, 4% glycerol, 2 mM $MgCl_2$, 0.08 mM EDTA, 0.04% NP-40, 50 μM ammonium iron(II) sulfate, 2 mM ascorbic acid, and 1 mM α-ketoglutarate at 37°C for one hour. For assessing repair of the dsDNA substrate, the antisense oligonucleotide was annealed to the labelled 3MeC oligonucleotide in a 3:1 molar ratio. For assessing the repair of the ssDNA substrate, the

antisense oligonucleotide was annealed as above after the repair reaction. Reactions were then digested with HpaII and analyzed by TBE-PAGE.

Statistical analyses

Data are presented as mean ± SD. Statistical analyses were performed using paired Student's *t*-test as indicated. A $p < 0.05$ value was considered significant, a $p < 0.001$ was considered highly significant. Growth rate analysis and proliferation assay was analyzed using the GraphPad Prism 5.03 software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- ALKBH3, a DNA/RNA dealkylating enzyme, associates with ASCC, an uncharacterized complex
- The largest subunit of ASCC, ASCC3, encodes a 3′-5′ DNA helicase
- ALKBH3 and ASCC3 promote dealkylation repair on double-stranded DNA
- Both ALKBH3 and ASCC3 are important for proliferation and dealkylation repair in specific tumor cell lines

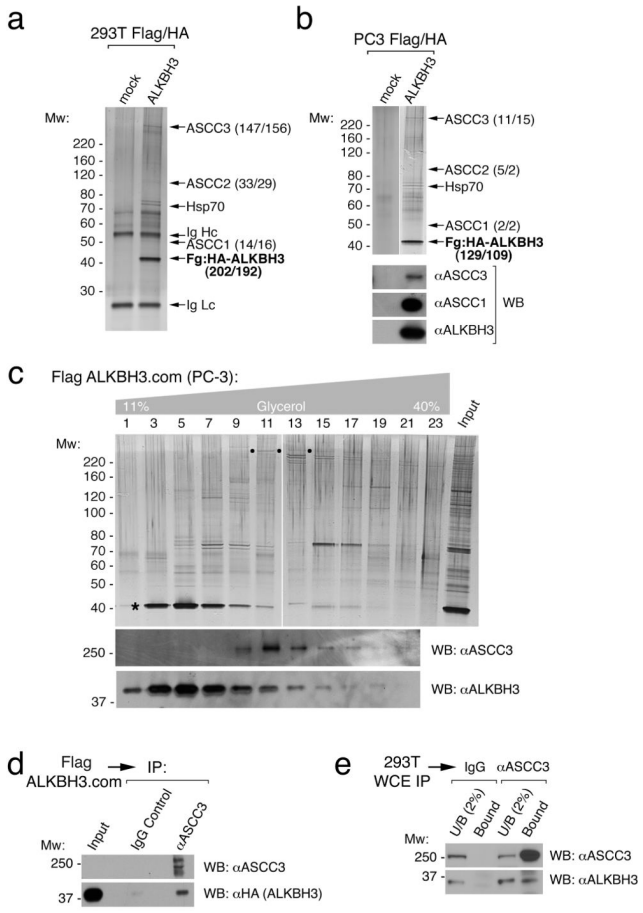

Figure 1.

ALKBH3 associates with the mammalian ASCC complex. **(a)** Silver staining of the ALKBH3 complex separated on 4-12% SDS-PAGE gel. The ALKBH3 complex was immunopurified from nuclear extract prepared from 293T cells stably expressing Flag-HA-ALKBH3. Subunits of the ASCC complex, as well as Hsp70, immunoglobulin heavy chain (Ig Hc) and light chain (Ig Lc) are indicated. Positions of molecular weight markers (Mw) are indicated (in KDa). Samples were analyzed by MS/MS twice; the total peptide numbers for each MS/MS run are shown in parentheses. **(b)** Silver staining of the ALKBH3 complex immunopurified from PC-3 nuclear extract stably expressing Flag-HA-ALKBH3. Major subunits of the ASCC complex ASCC3, as well as Hsp70 are indicated. **(c)** The Flag-purified PC-3 ALKBH3 complex (input) was fractioned by 11-40% glycerol gradient sedimentation. Fractions were visualized by silver staining (top) and Western blotted with the indicated antibodies (bottom). Black dots indicate the ASCC3 subunit; black asterisk indicates Flag-HA-ALKBH3. **(d)** Flag-purified ALKBH3 complex was immunoprecipitated with control IgG or ASCC3 antibody and blotted as indicated. **(e)** 293T whole-cell extract (WCE) was immunoprecipitated with control IgG or ASCC3 antibody and blotted as shown.

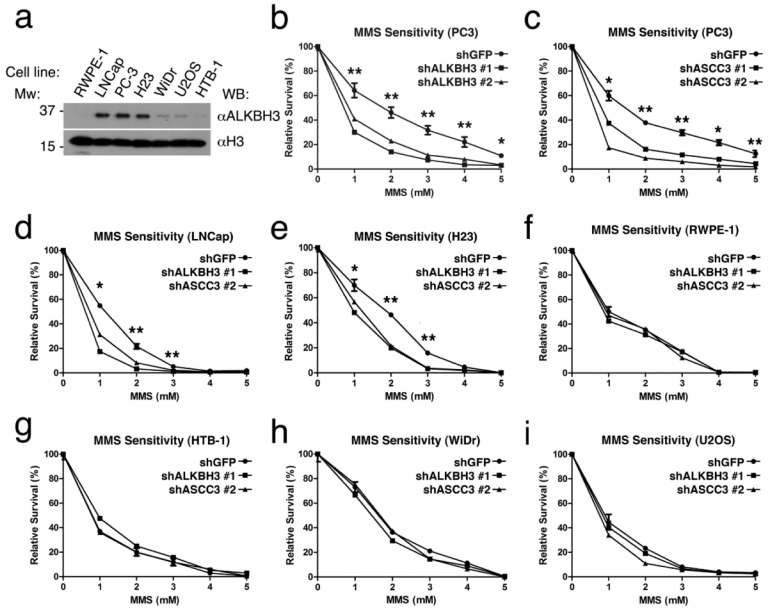

Figure 2.

Loss of ALKBH3 or ASCC3 results in MMS sensitivity in specific cell lines. **(a)** Whole cell extracts from the indicated cell lines were Western blotted using antibodies against ALKBH3 or histone H3. PC-3 **(b-c)**, LNCaP **(d)**, H23 **(e)**, RWPE-1 **(f)**, HTB-1 **(g)**, WiDr **(h)**, and U2OS **(i)** cells were infected with the indicated shRNAs. After selection, the infected cells were exposed to the indicated concentration of MMS for one hour and growth was assessed 48 hours later by MTS assay. All experiments were carried out in triplicate; Error bars indicate +/− S.D. * $p<0.05$; ** $p<0.001$.

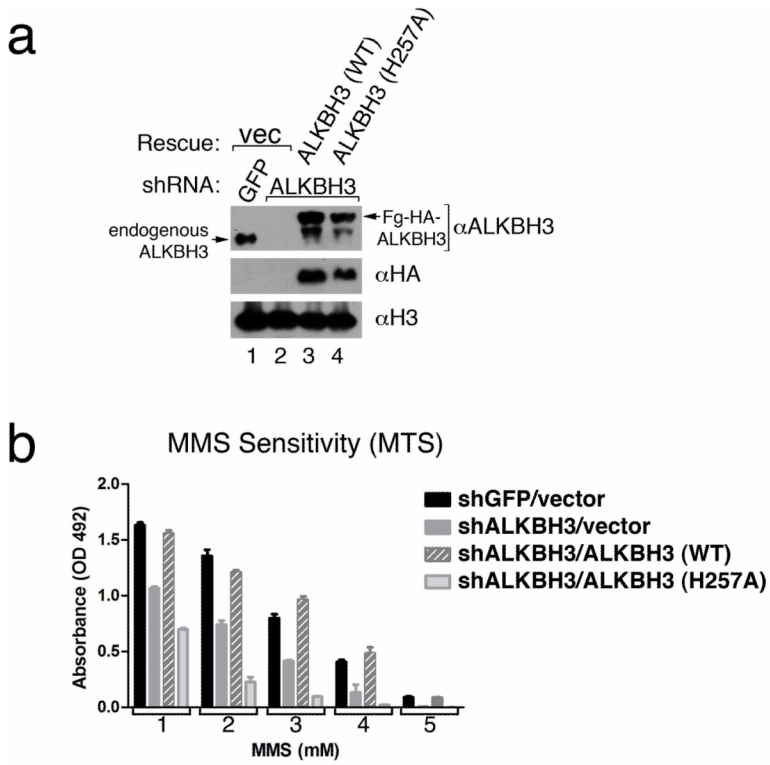

Figure 3.

Catalytic activity of ALKBH3 is important for resistance to alkylation damage in the PC-3 prostate cancer cell line. **(a)** PC-3 cells were infected with retroviral, shRNA resistant Flag-HA-ALKBH3 (WT or catalytically inactive H257A) or with control vector as well as the indicated shRNA vectors. After selection, whole cell extracts were analyzed by Western blot as shown. Cells from **(a)** were exposed to the indicated concentrations of MMS for one hour and assessed for growth by MTS after 48 hours **(b)**.

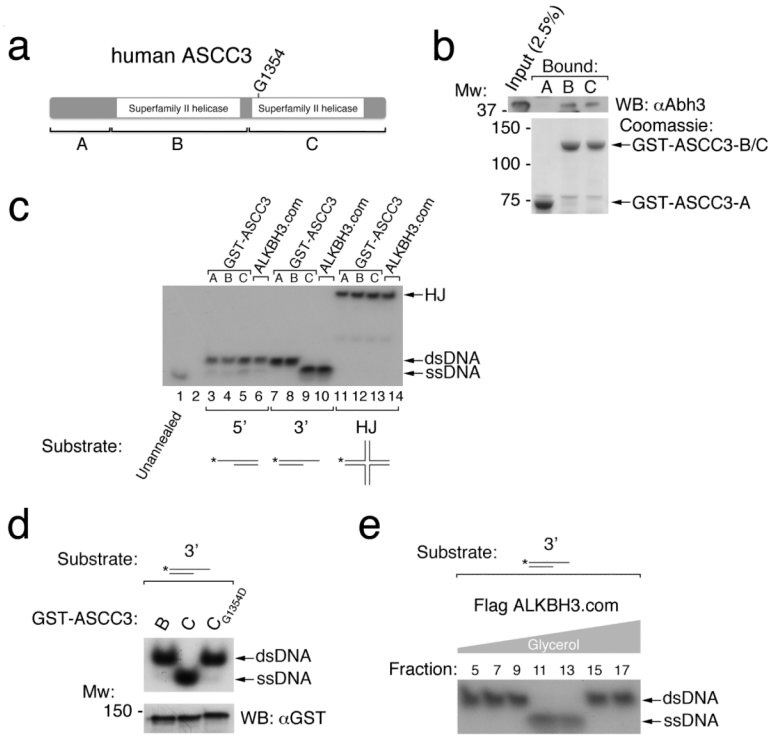

Figure 4.

ASCC3 binds directly to ALKBH3 and encodes a 3′-5′ DNA helicase. **(a)** Schematic overview of human ASCC3. A-C indicate the subcloned fragments of ASCC3 used below. **(b)** GST-tagged ASCC3-A, ASCC3-B, or ASCC3-C (3 μg each) was immobilized on glutathione-Sepharose, and binding of His_6-ALKBH3 (2 μg per reaction) was tested. After SDS-PAGE, the bound and input fractions were analyzed as indicated. **(c)** Helicase assays were performed using the indicated substrates in combination with recombinant ASCC3-A (7 μM per reaction; lane 3, 7, and 11), ASCC3-B (1.4 μM per reaction lane; 4, 8, and 12), ASCC3-C (0.4 μM per reaction; lane 5, 9, and 13), or Flag-ALKBH3 complex purified from PC-3 cells (10 μl per reaction; lane 6, 10, and 14). Unannealed 5′-labeled DNA substrate is shown (lane 1). **(d)** Helicase reactions were carried out with the 3′ overhang substrate using the indicated GST fragments of ASCC3, as well as the ATPase deficient G1354D mutant **(a)**. Protein loading was assessed by Western blot against GST (bottom). **(e)** Equal amounts of the glycerol fractions from Figure 1 (5-17) of the ALKBH3 complex were examined for helicase activity using the 3′ overhang substrate. *dsDNA*, double-stranded DNA; *ssDNA*, single-stranded DNA; *HJ*, Holliday junction.

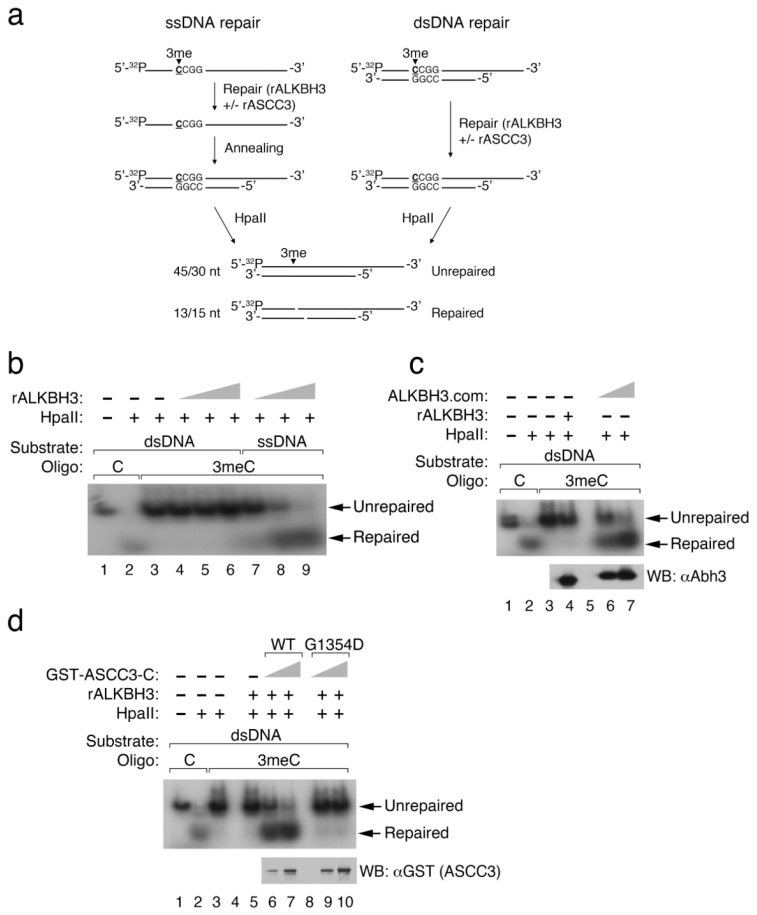

Figure 5.

ASCC3 promotes DNA dealkylation activity of ALKBH3 on dsDNA *in vitro*. **(a)** Schematic overview of reaction set-up used for the DNA dealkylation assays. 3me indicates the 3-methylated cytosine base in the methylation-sensitive HpaII restriction site. **(b)** Demethylation assay with recombinant His_6-ALKBH3 (13 nM, 50 nM, or 200 nM) using dsDNA (lanes 4-6) or ssDNA (lanes 7-9) probe containing the 3meC lesion. Unmethylated probe (C) (lanes 1 and 2) was used as a restriction digest control. **(c)** dsDNA 3meC repair using Flag-purified ALKBH3 complex (ALKBH3.com; 10-30 μl; lanes 6 and 7, respectively) or recombinant His_6-ALKBH3 (rALKBH3; 75 nM; lane 4). Western blot analysis using the ALKBH3 antibody is shown (bottom). **(d)** Introduction of wild-type (20-80 nM; lanes 6 and 7) but not catalytically inactive GST-ASCC3-C (lanes 9 and 10) promoted the repair activity of recombinant His_6-ALKBH3 (75 nM) on dsDNA substrates. Western blot analysis for GST is shown (bottom).

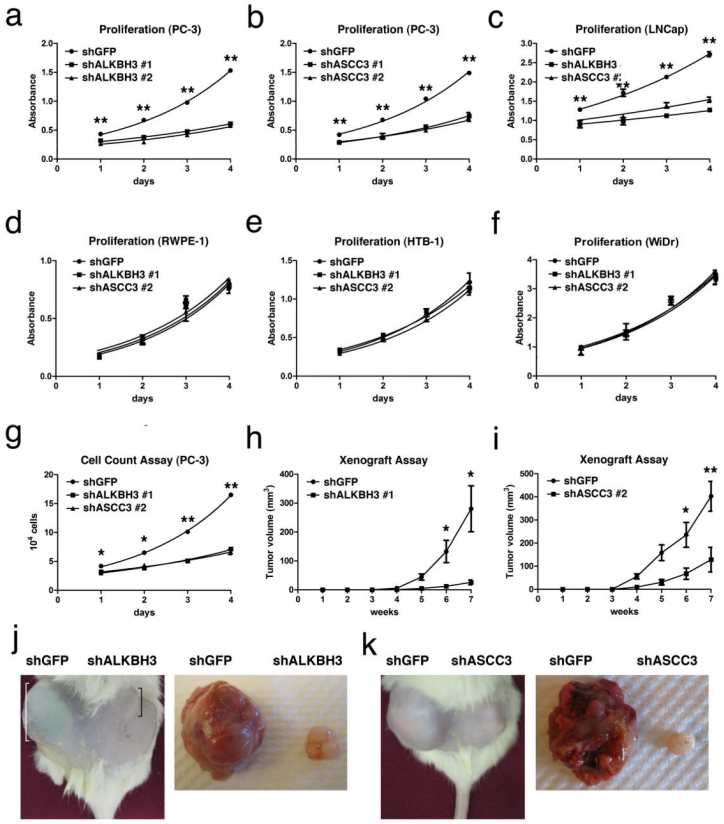

Figure 6.

Knockdown of ALKBH3 or ASCC3 results in reduced cell proliferation in tumor cell lines overexpressing ALKBH3. **(a)** Cell proliferation was assessed by MTS assay over 4 days after shRNA-mediated knockdown of ALKBH3 or ASCC3 in PC-3 **(a, b)**, LNCaP **(c)**, RWPE-1 **(d)**, HTB-1 **(e)**, and WiDr **(h)** cells. **(g)** Absolute cell number was counted after seeding shRNA infected PC-3 cells over 4 days. All experiments were carried out in triplicate. **(h, i)** PC-3 cells infected with control (GFP) shRNA or shRNA specific for ALKBH3 **(h)** or ASCC3 **(i)** were injected into the right or left flanks of castrated male SCID mice, respectively. Tumour growth was assessed as described in experimental procedures (n = 4 mice per group). **(j, k)** A representative mouse from each group at seven weeks is shown in the left panels; right panels show the whole tumor after excision. Error bars indicate +/− S.D. * $p<0.05$; ** $p<0.001$.

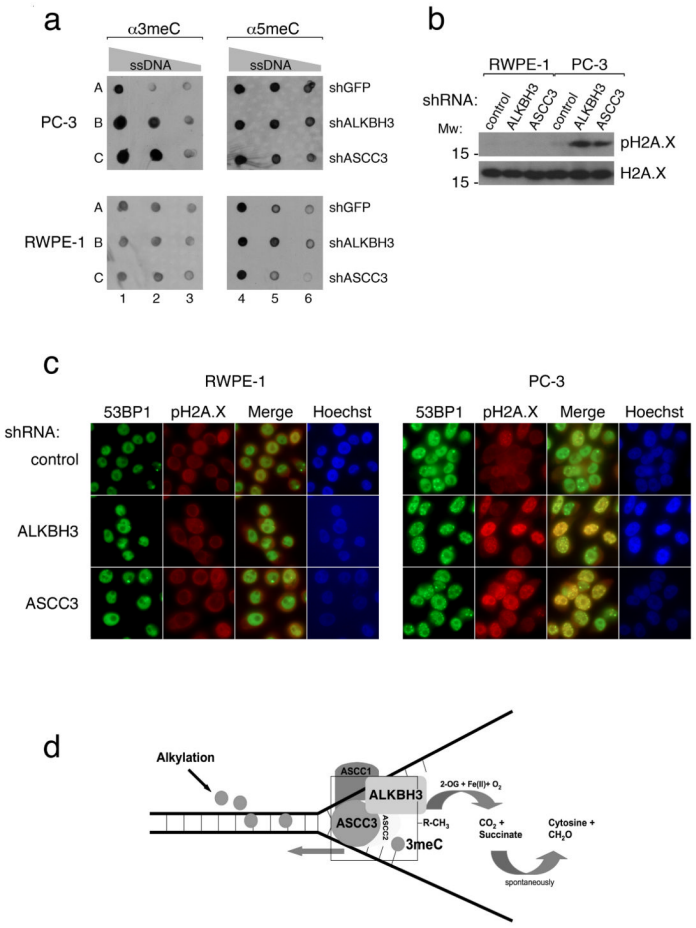

Figure 7.

Knockdown of ALKBH3 or ASCC3 causes 3-methylcytosine accumulation and activation of the DNA damage response in prostate cancer cells. **(a)** Detection of 3meC levels in the genomic DNA of PC-3 cells (top) or RWPE-1 cells (bottom) after control knockdown (shGFP; row A), ALKBH3 knockdown (row B), or ASCC3 knockdown (row C). After extraction and purification, serial dilutions of genomic DNA (150 ng, 75 ng, and 37.5 ng) was blotted and probed using an anti-3meC antibody (lanes 1-3). DNA loading was assessed by blotting the same amount of genomic DNA using an anti-5meC antibody (lanes 4-6). **(b)**. Knockdown of ALKBH3 or ASCC3 leads to formation of pH2A.X in the absence of exogenous damage in PC-3 cells. After infection with the indicated shRNA, whole cell extracts of the indicated cell lines were prepared and Western blotted using anti-H2A.X and anti-pH2A.X antibodies. **(c)** Cells treated as in **(b)** were processed for immunofluorescence using the indicated antibodies. **(d)** Schematic working model of the interplay of ALKBH3 and ASCC3 in alkylation repair; alklyation damage is indicated by grey dots.