The interaction of DNA repair factors ASCC2 and ASCC3 is affected by somatic cancer mutations

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The ASCC3 subunit of the activating signal co-integrator complex is a dual-cassette Ski2-like nucleic acid helicase that provides single-stranded DNA for alkylation damage repair by the α-ketoglutarate-dependent dioxygenase AlkBH3. Other ASCC components integrate ASCC3/AlkBH3 into a complex DNA repair pathway. We mapped and structurally analyzed interacting ASCC2 and ASCC3 regions. The ASCC3 fragment comprises a central helical domain and terminal, extended arms that clasp the compact ASCC2 unit. ASCC2–ASCC3 interfaces are evolutionarily highly conserved and comprise a large number of residues affected by somatic cancer mutations. We quantified contributions of protein regions to the ASCC2–ASCC3 interaction, observing that changes found in cancers lead to reduced ASCC2–ASCC3 affinity. Functional dissection of ASCC3 revealed similar organization and regulation as in the spliceosomal RNA helicase Brr2. Our results delineate functional regions in an important DNA repair complex and suggest possible molecular disease principles.
The human genome is constantly under assault by endogenous or exogenous DNA damaging agents. To ward off these insults, cells have evolved systems to recognize DNA damage, signal its presence and initiate repair processes. Among the diverse repair mechanisms, direct DNA repair processes represent efficient means to revert chemical changes to DNA and involve enzymes such as photolyases, alkyl-transferases or dioxygenases. Escherichia coli α-ketoglutarate-dependent dioxygenase, AlkB, homologs (AlkBH) are one class of important DNA repair factors that reverse N-alkyl lesions. Among the nine genase, AlkB, homologs (AlkBH) suggesting that the proteins affect stalled ribosomes. Indeed, the enzyme is thought to provide single-stranded DNA as a substrate for de-alkylation repair by AlkB-H3 (ref. 9). AlkB and alkylated nucleotides co-localize at nuclear foci upon alkylation damage stress, dependent on a coupling of ubiquitin conjugation to ER degradation (CUE) domain in ASCC2, which links DNA alkylation damage repair to upstream ubiquitin signaling via the RING finger protein 113A9. ASCC1 is cleared from these foci upon DNA alkylation damage and knockout of ASCC1 leads to loss of ASCC2 from the nuclear foci and increased cellular sensitivity to alkylating insults.

Both ASCC2 and ASCC3 have been linked to various human diseases. ASCC2 is upregulated in patients with rheumatoid arthritis11, and ASCC3 is upregulated in peripheral blood mononuclear cells from patients with lung cancer12,13. A role of ASCC3 in cancer development or progression is also suggested by the observation that knockdown of ASCC3 in a prostate cancer cell line suppresses cell proliferation.6 Moreover, the Catalog Of Somatic Mutations In Cancer (COSMIC v91, release date 07 April 2020; https://cancer.sanger.ac.uk/cosmic) lists cancers and non-functional ribosomal RNA decay28. Several lines of evidence implicate the human activating signal co-integrator complex (ASCC) in AlkBH3-mediated DNA repair. ASCC is composed of four subunits, ASCC1, ASCC2, ASCC3, and ASCC1/TRIP4 (refs. 7,8). ASCC3 is the largest subunit of ASCC and was characterized as a DNA helicase that unwinds DNA by translocating on one strand in 3'-to-5' direction. The enzyme is thought to provide single-stranded DNA as a substrate for de-alkylation repair by AlkB-H3 (ref. 9). ASCC and alkylated nucleotides co-localize at nuclear foci upon alkylation damage stress, dependent on a coupling of ubiquitin conjugation to ER degradation (CUE) domain in ASCC2, which links DNA alkylation damage repair to upstream ubiquitin signaling via the RING finger protein 113A9. ASCC1 is cleared from these foci upon DNA alkylation damage and knockout of ASCC1 leads to loss of ASCC2 from the nuclear foci and increased cellular sensitivity to alkylating insults.

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**Results**

**Experimental definition of a stable, minimal ASCC2–ASCC3 complex.** Full-length ASCC2 (ASCC2FL) and two fragments of ASCC3, encompassing the NTR (ASCC3NTR; residues 1–207), and helicase region (ASCC3HR; residues 401–2202), were obtained by recombinant expression in insect cells. Analytical size exclusion chromatography (SEC) showed that ASCC2 interacts with ASCC3NTR (Fig. 1a) but not with ASCC3HR (Fig. 1b), consistent with previous reports9. The complex assembled from ASCC2FL and ASCC3NTR failed to crystallize. To remove putatively flexible regions that may hinder crystallization, we subjected the ASCC2FL–ASCC3NTR complex to limited proteolysis and mapped stable fragments by mass spectrometric fingerprinting. Elastase digestion gave rise to an approximately 50 kDa fragment of ASCC2, containing the first 434 residues (ASCC2FL1–434), and an N-terminal, 207-residue fragment of ASCC3 (ASCC31–207), which maintained stable interaction in SEC (Fig. 1c). The ASCC2FL–ASCC3NTR complex was produced by recombinant co-expression in insect cells, purified and yielded diffracting crystals.

**Structure analysis and overall architecture.** The crystal structure of the ASCC2FL–ASCC3NTR complex was determined via the single wavelength anomalous dispersion (SAD) approach, using a complex reconstituted by SEC from seleno-methionine (SeMet)-derivatized ASCC21–434 produced in E. coli and ASCC31–207 produced in insect cells (Supplementary Fig. 1). The structure was refined at 2.7 Å resolution to Rwork/Rfree values of 20.4/24.7% with good stereochemistry (Supplementary Table 1). An asymmetric unit of the crystals contains one ASCC21–434–ASCC31–207 complex. In the final model, we traced residues 2–408 of ASCC21–434 activation, catalysis and disassembly. Based on the well-documented structural organization of Brt2 (refs. 38–39), ASCC3 is predicted to encompass a large N-terminal region (NTR; residues 1–400) and a helicase region (HR; residues 401–2202) containing a tandem array of two Ski2-like helicase cassettes. Each helicase cassette is predicted to encompass two RecA-like domains, a winged-helix domain and a Sec63 homology unit; the latter elements comprise sequential helical bundle or “ratchet”, helix-loop-helix and immunoglobulin-like domains. Brt2 is a subunit of the U4/U6•U5 tri-snRNP14,33. ATPase/helicase activities of Brt2 are tightly regulated both intrinsically and by interacting proteins33,36–39. The high sequence similarity between ASCC3 and Brt2, and the observation that ASCC3, like Brt2, interacts with a number of other proteins, suggest that ASCC3 might be regulated by similar principles.

Structural knowledge regarding ASCC subunits and their various interactions will be instrumental in further delineating the molecular mechanisms by which these proteins participate in diverse cellular functions and how they are linked to human diseases. While some interacting regions among ASCC subunits have been broadly mapped9,10, the structural basis of these interactions is presently unclear. Currently, only an unpublished NMR structure of the isolated ASCC2 CUE domain (PDB ID 2DFD) is known.

Here, we mapped precise interacting regions of the two largest ASCC subunits, ASCC2 and ASCC3, and determined a crystal structure of an ASCC2–ASCC3 complex comprising these portions. Guided by the structure, we delineated segments and residues that are critical for the ASCC2–ASCC3 interaction, which include a large number of residues that are altered by somatic cancer mutations. We also further investigated ATPase/helicase activities of ASCC3, and how they may be impacted by ASCC2.
with a gap between residues 216–226 representing a flexible loop. The ASCC31–207 model comprises residues 1–186 without gaps and two additional residues at the N-terminus that were retained after tag cleavage.

Within the complex, the structure of ASCC21–434 exhibits an irregularly structured N-terminal region (residues 2–50), and two similarly structured, helical sub-domains (sub-domain A, residues 51–214; sub-domain B, residues 241–406; Fig. 1d, e). Both
sub-domains are composed of eight α-helices (h1a-h8a/h1b-h8b; Fig. 1d). Each sub-domain, h1/h2, h4/h5, and h7/h8 form helical hairpins (beige/orange in Fig. 1d) that are connected by h3 and h6 (pink/magenta in Fig. 1d). h3a is significantly shorter than h3b (13 vs. 26 residues, respectively) and connected to h4a by a 13-residue, irregularly structured loop, as opposed to the single-residue connection between h3b and h4b. Furthermore, helices h7a and h8a are pried open compared to the h7b-h8b equivalents, due to helix h1b that wedges in between them (Fig. 1d).

The ASCC31–207 fragment folds into a central helical domain (residues 46–159) and extended arms at the N-termini and C-termini (N-arm, residues 1–45, C-arm, residues 160–186), with which it clasps the compact ASCC21–434 termini (N-arm, residues 197–207; Fig. 1d). The N-arm of ASCC31–207 forms three helices, h1, h2 and h3, preceded and connected by irregularly structured linkers along one flank of ASCC21–434 (Fig. 1d). The first helix (h1, residues 5–13) is embedded between helices h6a and h8a of ASCC21–434 sub-domain A and helix h1b of ASCC21–434 sub-domain B. Helix h2 (residues 15–21) runs across ASCC21–434 helix h3a. Helix h3 (residues 26–40) separates part of the N-terminal extension of ASCC21–434 between helices h1a and h3a of ASCC21–434 sub-domain A (Fig. 1f). The central helical region of ASCC31–207 consists of six helices (h4-h9). Helices h4, h5, h7, h8, and h9 are arranged in a circle around the central helix h6, in front of helix h2a of ASCC21–434 (Fig. 1f). The C-arm stretches in an extended, irregular conformation along the opposite flank of ASCC21–434, connecting sub-domains A and B of ASCC21–434 (Fig. 1f).

Interfaces between ASCC3 and ASCC2 are evolutionarily conserved. Structurally or functionally important regions in proteins are often evolutionarily conserved. We analyzed conservation of the ASCC21–434, ASCC31–207 contact regions using the ConSurf server.50 This analysis revealed that residues located at ASCC21–434, ASCC31–207 interface regions are highly conserved (Fig. 2). Residues 91, 93, 103, 163, and 251 of ASCC21–434 were assigned the highest conservation scores. These residues are located in helix h3a, h6a and h1b, and form a binding pocket for helix h1 of the ASCC31–207 N-arm (Fig. 2a, left). ASCC21–434 residues that form a binding surface for the ASCC31–207 C-arm are conserved to a lesser extent (Fig. 2a, right). Mirroring the conservation pattern on ASCC21–434, residues of the ASCC31–207 N-arm, in particular the first 16 residues, are significantly more conserved compared to other surface positions of the protein (Fig. 2b). This analysis suggests that interactions involving the N-arm of ASCC31–207 may be of particular importance for the formation of the ASCC21–434, ASCC31–207 complex, and that the ASCC21–434, ASCC31–207 interaction observed here is likely conserved in all organisms that contain these proteins.

The N-terminal arm of ASCC3 is essential for stable binding to ASCC2. To elucidate the relative importance of different regions of ASCC31–207 for interaction with ASCC21–434, we used structure-informed mutagenesis in combination with analytical SEC and isothermal titration calorimetry (ITC). First, we designed sequential N-terminal truncations of ASCC31–207 with or without the C-arm. ASCC3 fragments lacking C-terminal residues not visible in the structure (ASCC31–197) or additionally lacking the C-arm (ASCC31–161) maintained stable interaction with ASCC21–434 in SEC (Fig. 3a). By contrast, deletion of the N-arm (ASCC31–197) abolished stable binding to ASCC21–434 in SEC (Fig. 3b), while a partial N-arm deletion variant (ASCC31–197) still co-migrated with ASCC21–434 in SEC (Fig. 3c).

Quantifying binding affinities by ITC revealed a similar, yet more detailed, picture. ITC showed a $K_d$ of 3.5 nM for the interaction of the complete ASCC3NTB and ASCC2FL (Fig. 3d). ASCC31–197 (lacking the C-terminal 10 residues of ASCC31–207 but containing all ASCC3 residues with well-defined electron density in the ASCC21–434, ASCC31–207 complex structure) and ASCC21–434 interacted with a similar affinity ($K_d$ of 3.8 nM), suggesting that the fragments contained in our crystal structure encompass the entire ASCC2–ASCC3 interacting regions (Fig. 3e). Deletion of the entire C-arm of ASCC31–207 (ASCC31–161) led to an approximately 14-fold decreased affinity ($K_d = 47.7$ nM; Fig. 3f). Truncation of the N-terminal 15 residues that form helix h1 in the N-arm of ASCC31–197 reduced affinity to ASCC21–434 by more than two orders of magnitude ($K_d = 483.0$ nM; Fig. 3g). Lack of the entire N-arm of ASCC31–197 (ASCC31–161) completely abrogated the interaction, as no signal was detected in ITC measurements (Fig. 3h). Together, the above results indicate that the N-arm, and in particular the first 15 residues, of ASCC31–207 are essential for a stable binary interaction with ASCC21–434, consistent with its high degree of evolutionary conservation. The C-arm of ASCC31–207 contributes to the interaction with ASCC21–434 but it is not essential for the proteins to maintain a stable complex in SEC, consistent with a reduced but still high level of evolutionary conservation of the C-arm.

We next tested whether the interaction pattern observed using recombinant proteins in vitro also applies to the ASCC2–ASCC3 interaction in living cells. Expression constructs encoding C-terminally flag-tagged ASCC3FL and stepwise N-terminally truncated fragments (ASCC316-end, ASCC312-end, ASCC3207-end and ASCC3401-end) were used to generate stably transfected HEK293 Flp-In cell lines for the tetracycline-inducible expression of these proteins. After induction, flag-tagged ASCC3 or fragments were captured on anti-flag beads and co-precipitation of ASCC2 was monitored by western blotting. In line with our in vitro interaction mapping, ASCC3 variants with increasing N-terminal deletions co-precipitated stepwise reduced amounts of ASCC2 (Fig. 3i). ASCC3 variants lacking 15, 41, 206, or 400 N-terminal residues, co-precipitated 73, 40, 26, or 16%, respectively, of the amount of ASCC2 associated with full-length ASCC3 (Fig. 3j). While based on our structural and mutagenesis data truncation of the N-terminal 206 or 400 residues of ASCC3 should abrogate direct ASCC2–ASCC3 interactions, ASCC3207-end and ASCC3401-end most likely still pulled down reduced amounts of ASCC2 (Fig. 3i, j), because other ASCC subunits also mediate indirect ASCC2–ASCC3 interactions in vivo.

ASCC2 and ASCC3 residue substitutions found in human cancers cluster at interfaces and lead to reduced affinities. 223 and 652 somatic nonsense, missense or frame shift mutations in human cancer cell or tissue samples have been mapped to the asc2 and asc3 coding regions, respectively (https://cancer.sanger.ac.uk/cosmic). 123 and 95 of these mutations affect residues in ASCC21–434 and ASCC31–207, respectively. Strikingly, 16 of the missense mutations and three nonsense mutations affect residues E53, R58, T60, D65, L70, R96, Y97, D103, or R121 in ASCC21–434, and 17 or one, respectively, of these mutations affect residues R5, R11, S12, D28, R33, K165, or E181 in ASCC31–207, which all constitute direct contact points between the proteins in our crystal structure (Fig. 4a). Many of the remaining point mutations map to, and are expected to disturb, the globular portions of the proteins. In addition, there are three frameshift mutations that would affect almost the entire ASCC21–434 region or large parts thereof (E30, F124, G171 in ASCC2; Fig. 4a). 25 frameshift mutations map to residues F163 and G164 in ASCC3, affecting the entire C-arm of ASCC31–207 (Fig. 4a).

We thus surmised that many residue substitutions of ASCC21–434 or ASCC31–207 found in human cancers affect the
ASCC2–ASCC3 affinity. To test this hypothesis, we conducted comparative ITC analyses using ASCC21–434 and peptides representing the N-terminal 22 residues of ASCC3 exhibiting wild-type (WT) sequence or residue substitutions R5G, R5C, R5H, R5L, R11C, or R11H. R5 residue substitutions are found in large intestine and lung adenocarcinomas, as well as in cervical and esophageal squamous cell carcinomas, while the R11 residue substitutions occur in endometrioid carcinoma and large intestine adenocarcinoma (https://cancer.sanger.ac.uk/cosmic). In our experiments as the high affinity observed in ITC between ASCC21–434 and ASCC31–434 (D103 of ASCC2 is positioned at the N-terminus of helix h1 and interacts with D103 of ASCC21–434) weakened the interaction with ASCC3 variants (see below) with a Kd of 2.0 µM (Fig. 4b). We used ASCC3 peptides in these experiments as the high affinity observed in ITC between ASCC21–434 and ASCC31–197 may mask differences due to single residue substitutions.

WT ASCC31–22 bound to ASCC21–434 with a Kd of 2.0 µM (Fig. 4c). ASCC31–22 peptides bearing R5L or R5G substitutions weakened the interaction with ASCC21–434 approximately eight-fold and eleven-fold, respectively (Fig. 4d, e). ASCC31–22 peptides comprising R5H or R5C substitutions showed more than 20-fold reduced affinities compared to WT (Fig. 4f, g). R11H or R11C substitutions in ASCC31–22 completely eradicated binding to ASCC21–434 (Fig. 4h, i). These observations are consistent with the notion that reduced affinity to ASCC2 represents a means by which the R5 and R11 residue substitutions in ASCC3 contribute to cancer phenotypes.

The N-terminal cassette is an active helicase unit in ASCC3. ASCC3 bears close resemblance to the splicosomal RNA helicase, Brr2. Both ASCC3 and Brr2 contain an approximately 400-residue NTR followed by two Ski2-like helicase cassettes with identical domain composition (Fig. 5a, left). It is well documented that the N-terminal Ski2-like helicase cassette constitutes the active helicase unit in Brr2, while the C-terminal cassette lacks ATPase and helicase activities. The reverse situation has been reported for human ASCC3; in contrast to an inactive isolated N-terminal cassette construct, an isolated C-terminal cassette construct was found to be active in DNA duplex unwinding. As we did not perceive alterations in conserved helicase motifs in the N-terminal cassette that would obviously preclude helicase activity (Fig. 6a), we revisited the question of helicase activity of the ASCC3 helicase cassettes.

While we were able to produce a recombinant ASCC3 fragment encompassing the N-terminal cassette (ASCC3NC; residues 401–1300), a fragment containing only the C-terminal unit could not be produced in soluble form. To test helicase activities, we pre-incubated ASCC3 variants (see below) with a 12-base pair DNA duplex containing a 31-nucleotide single-stranded overhang, and bearing a fluorophore (Alexa Fluor 488) on the 3′-terminus of the short strand and a quencher (Atto 540 Q) on the 5′-terminus of the long strand (Fig. 5b). As a control, we first tested the assay with Brr2 variants and an analogous RNA duplex. Fast mixing of Brr2FL or Brr2HR (residues 395–2129; a truncated version of Brr2 lacking most of the auto-inhibitory NTR, largely equivalent to ASCC3HR) pre-incubated with the RNA substrate and ATP in a stopped-flow device led to time-dependent increases in the Alexa Fluor 488 fluorescence, indicating RNA duplex unwinding (Fig. 5c). Unwinding occurred at a significantly higher rate with Brr2HR than with Brr2FL (Fig. 5c), showing that the stopped-flow/fluorescence setup can reliably monitor differences in helicase activity between alternative single-cassette helicase constructs. As expected, ASCC3HR

![Image](https://example.com/image.png)
efficiently unwound a DNA duplex with 3'-single stranded overhang, but not a duplex with 5'-single stranded overhang (Fig. 5d), and preferentially used ATP for DNA unwinding (Fig. 5e). Surprisingly, however, in our hands, ASCC3NC also strongly interfered with helicase activity. To further test the contributions of the N-terminal and C-terminal cassettes to the helicase activity of a dual-cassette ASCC3 construct, we individually substituted the corresponding lysine and aspartate residues in the N-terminal cassette (K505, D611), in the helicase activity of a dual-cassette ASCC3 construct, we individually substituted the corresponding lysine and aspartate residues in the N-terminal cassette (K505, D611), in the

Nucleic acid helicases contain a number of conserved, functionally important sequence motifs in their RecA domain cores (Fig. 6a). In particular, a lysine in motif I is required for ATP binding, while an aspartate in motif II is crucial for coordinating a magnesium ion to trigger the hydrolysis of ATP (Fig. 6a), and substitutions of these residues are expected to strongly interfere with helicase activity. To further test the contributions of the N-terminal and C-terminal cassettes to the helicase activity of a dual-cassette ASCC3 construct, we individually substituted the corresponding lysine and aspartate residues in the N-terminal cassette (K505, D611), in the

**Fig. 3 Interaction analysis.** a-c SDS-PAGE analysis monitoring SEC runs of the indicated mixtures of proteins. d-h ITC runs monitoring the interaction between the indicated pairs of proteins. Deduced $K_d$ values are listed as means ± SD for runs for which affinities could be quantified. i Western blot monitoring pulldown of ASCC2 by the indicated ASCC3 constructs from cell extracts. j Quantification of the data shown in i. Columns represent means relative to ASCC3FL of $n = 2$ independent experiments, using the same biochemical samples. Open circles, individual measurements.
C-terminal cassette (K1355, D1453) or in both cassettes of ASCC3HR with alanine or asparagine, respectively. We verified the intended mutations by sequencing the final generation of baculoviruses used for production of the ASCC3HR variants (Supplementary Fig. 3). ASCC3HR variants that contained mutations in motifs I or II of the N-terminal cassette had strongly reduced or no detectable helicase activities (Fig. 6b). By contrast, ASCC3HR variants that contained residue substitutions K1355N or D1453A within the C-terminal cassette retained the activity of WT ASCC3HR (Fig. 6c). Again, gel-based unwinding assays using D611A and D1453A variants of ASCC3HR were consistent with these results (Supplementary Fig. 2). Also consistent with these findings, DNA helicase activities of the K505N/K1355N and D611A/D1453A double variants were

Fig. 4 Interface residues in ASCC2 and ASCC3 affected by somatic cancer mutations. a Orthogonal views on the ASCC21-434, ASCC31-207 structure, highlighting interface residues and frameshift positions affected by somatic cancer mutations. ASCC21-434, beige; ASCC31-207, lime green; ASCC2 residues, brown sticks; ASCC3 residues, dark green sticks; first positions affected by frameshift mutations, red. Orientations of the panels as in Fig. 1d.f. b Details of the interaction networks involving cancer-related residues D103 of ASCC2, as well as R5 and R11 of ASCC3. Dashed lines, salt bridges. Rotation symbol, view relative to Fig. 1d.f, left. c-i ITC runs monitoring the interaction of ASCC21-434 with the indicated ASCC31-22 peptide variants. Deduced $K_d$ values are listed as means ± SD for runs for which affinities could be quantified.
**Fig. 5 Unwinding assays.**  
(a) Comparison of the structure of full-length yeast Brr2 (left) and the present ASCC2\(^{1-434}\)–ASCC3\(^{1-207}\) complex (right) after superposition of the plug/plug-like domains (blue). Human Brr2 in the U4/U6●U5 tri-snRNP or in the pre-catalytic spliceosome exhibits a plug domain very similar to yeast Brr2 blocking RNA access\(^{42,43}\) (PDB IDs 3JCR, 6QW6, 6QX9). NC, N-terminal cassette; CC, C-terminal helicase cassette.  
(b) Experimental setup for stopped-flow/fluorescence-based unwinding assays. Gray sphere, helicase; star symbol, fluorophore (Alexa 488); Q, quencher (Atto 540 Q).  
(c) Stopped-flow/fluorescence-based assays monitoring unwinding of a 3′-overhang RNA by Brr2\(^{FL}\) or Brr2\(^{HR}\), showing that auto-inhibition, which has been documented for Brr2 using gel-based unwinding assays\(^{33}\), can be readily detected using the present experimental setup.  
(d) Stopped-flow/fluorescence-based assays monitoring unwinding of DNA bearing a 3′-overhang (3′-ovh), and lack of unwinding of a 5′-overhang (5′-ovh) DNA by ASCC3\(^{HR}\) upon addition of ATP.  
(e) Nucleotide preference of ASCC3\(^{HR}\) in unwinding a 3′-overhang DNA.  
(f) Stopped-flow/fluorescence-based assays monitoring unwinding of 3′-overhang DNA by ASCC3\(^{NC}\) compared to ASCC3\(^{HR}\) using ATP.  
(g) Stopped-flow/fluorescence-based assays monitoring unwinding of a 3′-overhang DNA by the indicated ASCC3 constructs in the absence or presence of ASCC2 using ATP. Insets, side-by-side presentation of the largely overlapping curves.  
(h) Stopped-flow/fluorescence-based assays monitoring unwinding of a 3′-overhang DNA by ASCC3\(^{HR}\) alone or in the presence of ASCC3\(^{NTR}\) or ASCC3\(^{42-161}\) using ATP. Insets, side-by-side presentation of the largely overlapping curves. FL/FR/NC/(42–161), ASCC3 variants as defined in the text.
almost fully abrogated (Fig. 6d). These results suggest that, contrary to previous observations\(^8\) and similar to Brr2 (ref. \(^32\)), the N-terminal cassette of ASCC3 is an active helicase in vitro. As the ASCC3\(^{NC}\) helicase activity was reduced compared to that of the N-terminal cassette of ASCC3 is an active helicase in vitro. As for 36 pairs of common C\(^{Brr2}\) plug domain (root-mean-square deviation [rmsd] of 2.7 Å related yeast Brr2 (ref.\(^33\)), as well as in human Brr2 in the U4/U5 tri-snRNP or in the pre-catalytic spliceosome\(42,43\), the NTR or part of it can fold back onto the helicase region, with the plug-like domain extend along ASCC2 1\(^{61}\), did not alter ASCC3\(^{HR}\) helicase activity (Fig. 5f), Supplementary Fig. 2) it remains to be seen whether the C-terminal cassette is inactive but stimulates helicase activity of ASCC3\(^{NC}\), as has been observed for Brr2 (ref. \(^32\)), or whether ASCC3\(^{CC}\) is an active helicase as well that contributes to the overall activity of ASCC3\(^{HR}\).

ASCC2 does not influence the helicase activity of ASCC3. The Brr2 NTR contains helical “plug” and PWI-like domains connected by extended, intrinsically disordered regions\(33,41\). In isolated yeast Brr2 (ref. \(^33\)), as well as in human Brr2 in the U4/U6•U5 tri-snRNP or in the pre-catalytic spliceosome\(42,43\), the NTR or part of it can fold back onto the helicase region, with the plug domain blocking substrate RNA loading (Fig. 5a, left), thereby auto-inhibiting Brr2 ATPase and helicase activities. Having established a similar functional organization of the helicase regions of ASCC3 and Brr2, we sought to investigate whether ASCC3 is also auto-regulated via its NTR.

While the overall sequence identity between human ASCC3 and human Brr2 is around 41%, the sequence identity is lower for their NTRs, around 20%. Despite the lower level of sequence conservation, structural comparison of ASCC3\(^{1–207}\) with the NTR of yeast and human Brr2 revealed that a central, four-helix portion of ASCC3\(^{1–207}\) (residue 59–142) closely resembles the Brr2 plug domain (root-mean-square deviation [rmsd] of 2.7 Å for 36 pairs of common Ca atoms; Fig. 5a). However, in our crystal structure, the ASCC3\(^{1–207}\) arms neighboring the plug-like domain extend along ASCC2\(^{1–434}\) (Fig. 5a, left); thus, when bound to ASCC2, these regions would not be available to fold back on the ASCC3 helicase region.

The helicase activity of ASCC3\(^{FL}\) was significantly reduced compared to ASCC3\(^{HR}\), consistent with similar auto-inhibition as for Brr2 (Fig. 5g). When added in trans, ASCC3\(^{NTR}\) or the plug domain-containing fragment, ASCC3\(^{42–161}\), did not alter ASCC3\(^{HR}\) helicase activity (Fig. 5h), indicating that the NTR has to be covalently connected to the helicase region to elicit the auto-inhibitory effect. Addition of ASCC2 did not influence the helicase activity of ASCC3\(^{FL}\). Due to the high affinities quantified for the ASCC2\(^{FL}\)-ASCC3\(^{NTR}\) and ASCC2\(^{1–434}\), ASCC3\(^{1–197}\) interactions (Fig. 3d, e), we consider it unlikely that the NTR is sequestered from ASCC2 by interacting in a mutually exclusive manner with the helicase region. Instead, the plug-like domain in ASCC3 may still be able to occupy an inhibitory position when associated with ASCC2.

Discussion

In this study, we have delineated interacting regions of the ASCC2 and ASCC3 subunits of the ASCC DNA repair machinery, and elucidated the structural basis for this interaction. Guided by our structural results, we identified regions and residues of the two proteins that significantly contribute to stable complex formation. The importance of regions for the interaction scales with the degree of their evolutionary conservation. Moreover, we observed that a large number of ASCC2 and ASCC3 residue substitutions encoded by somatic mutations in cancers map to...
ASC2–ASC3 interface regions and that selected, cancer-related residue substitutions in ASC3 lead to reduced ASC2–ASC3 affinity. We also conducted in vitro enzymatic analyses to better characterize the ASC3 nucleic acid helicase, showing (i) that the N-terminal cassette represents an active helicase, (ii) that helicase activity in the C-terminal cassette is dispensable for the activity of the full-length protein, (iii) that helicase activity in ASC3 is auto-inhibited by the NTR, and (iv) that interaction with ASC2 does not strongly influence ASC3 DNA unwinding activity in vitro.

ASC3 and Br2 both belong to a sub-group of the Ski2-like family of SF2 helicases with a tandem array of complex helicase units. The helicase regions of ASC3 and Br2 seem to be organized in an analogous fashion, comprising an active N-terminal and an inactive, or at least largely dispensable, C-terminal helicase cassette. Consistent with this notion, an ASC3 variant bearing an ATPase-disrupting substitution in the C-terminal helicase cassette, but not in the N-terminal cassette, can rescue a ribosome-poly-A readthrough phenotype elicited by ASC3 knockdown25. Likewise, in the yeast ASC3 homolog, Slt1p, residue substitutions in the N-terminal helicase cassette can abrogate ribosome quality control function in vivo27.

Several proteins have been identified that directly bind to the helicase region of Br2 and modulate its helicase activity, including a C-terminal Iab1-like domain of the Prp8 protein26 and a largely intrinsically unstructured protein, FBP21 (ref.38). ASCC1 interacts with the helicase region of ASC3 but not with the NTR10. It will be interesting to see in future studies, which of the cassettes it interacts with, whether it modulates the ASC3 helicase upon binding and whether similar molecular principles as in the case of Br2 and its protein co-factors apply to such putative regulation of ASC3.

Our structural analysis revealed that ASC3 and Br2 share at least some structural organization in their NTRs as well. Both contain a plug/plug-like domain close to their N-termini (residues 59–142 in human ASC3, 113–192 in yeast Br2, 107–180 in human Br2). In Br2, the plug domain contributes to auto-inhibition of the isolated enzyme25 and our results suggest similar auto-inhibition in isolated ASC3C. Based on our crystal structure, regions neighboring the ASC3C plug-like domain are expected to be guided away from the ASC3C helicase region when bound to ASC2, however, ASC2C did not alleviate ASC3C auto-inhibition. ASC3C auto-inhibition may, therefore, be predominantly mediated via the plug-like domain, which remains accessible in complex with ASC2C, occupying a position on the helicase region where it competes with DNA substrate loading. Still, our data suggest that a covalent connection of the plug-like domain to the helicase region is required for auto-inhibition. Thus, the general principle of auto-inhibition via the NTR seems to be conserved between ASC3 and Br2, but details of the mechanisms may differ.

Correlation of the evolutionary conservation of ASC2–ASC3 interfaces and the contributions of these regions to a stable interaction underscore the functional importance of the complex we structurally analyzed. As the two proteins seem to participate together in various ASC functions (transcription co-activation, DNA repair, ribosome-dependent protein quality control, viral defense), we expect that all of these functions depend on the observed ASC2–ASC3 interaction.

We observed an extraordinary number of residues affected by somatic cancer mutations at ASC2–ASC3 interfaces, and found that some cancer-related residue substitutions lead to reduced ASC2–ASC3 affinity. Based on these observations and previous insights into the role of ASC components in DNA alkylation damage repair, we suggest that reduced ASC2–ASC3 affinity might contribute to malignant transformation. As DNA alkylation damage can occur non-enzymatically44,45, the increased metabolic activity of cancer cells may lead to a higher level of metabolic DNA damage46, rendering cancer cell proliferation dependent on systems that can repair excessive DNA damage. Indeed, ASC3 is over-expressed in several cancers and ASC3 knockdown, which would be expected to lead to less efficient AlkBH3-mediated DNA alkylation repair, has been shown to negatively impact tumor cell proliferation in culture and xenograft models8,13. Other components of ASCC seem to contribute to efficient ASCCC/AlkBH3-mediated DNA alkylation damage repair by establishing a complex DNA damage signaling and repair pathway28,19. Specifically, ASCC1 appears to promote co-localization of ASCC2 and ASCC3 at nuclear foci during alkylation damage, and loss of ASCC1 leads to increased alkylation sensitivity10. Proper co-localization of ASCC2 and ASCC3, therefore, appears to be essential for efficient DNA alkylation damage repair. Similar to ASCC1 knockdown, reduced ASCC2–ASC3 affinity, as elicited by several somatic cancer mutations, may influence ASCC2–ASC3 co-localization at nuclear foci, leading to increased DNA alkylation damage. This model does not invoke a direct influence of ASCC2 on ASCC3 helicase activity, consistent with our findings. While reduced DNA alkylation damage repair due to reduced ASCC2–ASC3 affinity would be expected to negatively impact cancer cell proliferation, DNA alkylation can represent mutagenic lesions44,45. Thus, reduced ASCC2–ASC3 interaction may contribute to the initial development of cancer cell phenotype, as increased DNA alkylation damage may lead to transforming mutations.

**Methods**

**Molecular cloning.** DNA regions encoding the proteins/protein regions of interest were PCR-amplified from synthetic genes (GeneArt), optimized for expression in *Trichoplusia ni* cells. Supplementary Tables 2 and 3 list sequences of PCR primers and synthetic genes, respectively. DNA regions encoding ASCC3F, ASCC3NTR, ASCC3H, ASCC3C, ASCC1, ASCC2 or full-length ASCC2 were cloned into a modified pFL vector under control of the very late polyhedrin promoter, which directed the synthesis of fusion proteins with TEV-cleavable N-terminal His6-tags. A DNA fragment encoding ASCC21–434 was cloned into the pUCDM vector and Cre-recombined with ASCC320–207-encoding pFL. The QuikChange II XL Site-Directed Mutagenesis Kit (Agilent) was used to introduce mutations that give rise to ASCC3HR variants K50N, K135N, D61A, D143A, K585N, K135N, and D611A-D143A. The expression cassettes were integrated into the MultiBac baculoviral genome via Tin transposition within a lacZα gene, allowing selection of recombinants by blue/white screening. Recombinant bacterial artificial chromosomosomes (BACs) were isolated from the bacterial hosts.

**Protein fragments encoding ASCC2, ASCC3 and ASCC3 variants.** A DNA fragment encoding ASCC21–207, ASCC31–192, ASCC31–161, ASCC3C1–192, and ASCC2C1–434 were PCR-amplified from the pFL vectors encoding ASCC3NTR or full-length ASCC2. These fragments were inserted into the pETM-11 vector (EMBL, Heidelberg) that guides production of proteins with TEV-cleavable N-terminal His6-tags. All constructs were confirmed by sequencing.

**Protein production and purification.** ASCC21–434 was co-produced with ASCC31–207 in High Five cells. For virus production, the isolated BAC DNA was transfected into S9 cells in a six-well plate. After 68 h, the supernatant containing the initial virus (V0) was collected and used to infect a 50 ml suspension culture of *Trichoplusia ni* cells. Initial virus particles (POIs) and for the ASCC21–207 protein production, the final virus (V0) was collected and used to infect a 50 ml suspension culture of *Trichoplusia ni* cells. Production of the intended ASCC3HR variants was obtained by auto-induction in cells grown in PASM-5052 medium48, which contained 10 µg/mg of chloramphenicol, allowing selection of *E. coli* BL21 (DE3) cells transformed with the corresponding pETM-11 vectors, cultivated in auto-inducing medium at 18 °C and harvested at an OD₅₆₀ of ~10. SeMet-labeled ASCC21–434 protein was produced by auto-induction in cells grown in PASM-5052 medium48, which contained 10 µg/mg of unlabeled methionine and 125 µg/ml of SeMet. Cells were harvested by centrifugation and stored at –20 °C.

The same purification protocol was used for all individual proteins of interest (POIs) and for the ASCC21–434, ASCC31–207 used for crystallization, unless otherwise specified. Cell pellets were re-suspended in lysin buffer (20 mM HEPES-NaOH, pH 7.5, 500 mM NaCl, 10 mM imidazole, 1 mM DTT, 8.6% [v/v] glycerol), supplemented with protease inhibitors and lyzed by sonication using a Sonopuls Ultrasonic Homogenizer HD (Bandelin). After centrifugation and filtration, the soluble fraction was incubated with Ni²⁺-NTA beads for 1 h at 4 °C, and loaded into a gravity flow column. After extensive washing of the beads with lysis buffer,
the POI was eluted using lysis buffer containing 400 mM imidazole. The His6tag tags of the POI were cleaved off during overnight dialysis into lysis buffer without imidazole. Lysis buffer containing 400 mM imidazole was loaded on a fresh Ni2+-NTA gravity flow column to remove the His6tag, uncleaved protein and the His-tagged protease. For crystallization, the flow-through was further purified by SEC on a Superdex 200 10/60 GL column (GE Healthcare) in 20 mM HEPES-NaOH, pH 7.5, 100 mM NaCl, 1 mM EDTA. For other studies, the flow-through was purified by SEC on a Superdex 200 10/60 GL column in 20 mM HEPES-NaOH, pH 7.5, 250 mM NaCl, 1 mM EDTA, 5% (v/v) glycerol. For ASCC3NTR, the SEC step was omitted.

**Limited proteolysis.** 5.7 µg of purified ASCC2FL–ASCC3NTR complex were incubated with increasing amounts (0.0015, 0.013, and 0.13 µg) of different proteases in 20 mM HEPES-NaOH, pH 7.5, 250 mM NaCl, 1 mM DTT, 5% (v/v) glycerol at 4°C overnight. The reactions were stopped by adding SDS-PAGE loading buffer, and samples were separated by SDS-PAGE. Bands of interest were analyzed by gel trypsin digestion and mass spectrometric fingerprinting.

**Crystallographic procedures.** Unmodified or Semet-modified ASCC2–434, ASCC2–237 complex was crystallized by the hanging drop vapor diffusion technique using 20–30 mg/ml protein plus 1 µl reservoir solution that contained 0.1 M MES-NaOH, pH 6.5, 15% (w/v) polyethylene glycol 3350, 7% (v/v) glycerol in liquid nitrogen. Diffraction data were collected at beamline 14.1 of the BISSY II storage ring (Helmholtz-Zentrum Berlin, Germany) at 100 K. Diffraction data were processed with XDS49. The structure of ASCC2–434–ASCC3–207 complex was solved by the SeMet SAD strategy using phenix.autosol50. An initial model was calculated using phenix.autobuild34. The model was completed through alternating rounds of manual model building using Coot22 and automated refinement using phenix.refine33. Data collection, structure solution and refinement statistics are listed in Supplementary Table 1. Structure figures were prepared using PyMOL (Version 1.8 Schrödinger, LLC).

**Analytical size exclusion chromatography.** Proteins were produced and individually purified. 1 µmol ASCC2–434 was mixed with 2 µmol of ASCC3–161, ASCC2–237 or ASCC3–197 in 20 mM HEPES-NaOH, pH 7.5, 100 mM NaCl, 1 mM DTT to form the same volume of 50 µl. The mixtures were incubated overnight in 20 mM HEPES-NaOH, pH 7.5, 250 mM NaCl, 1 mM EDTA, 5% (v/v) glycerol at 4°C overnight. The reactions were stopped by adding SDS-PAGE loading buffer, and samples were separated by SDS-PAGE. Bands of interest were analyzed by gel trypsin digestion and mass spectrometric fingerprinting.

**Isothermal titration calorimetry.** ITC analyses were conducted on an iTC200 instrument (MicroCal). Proteins were dialyzed against ITC buffer (20 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 1 mM DTT, 5% (v/v) glycerol at 4°C) overnight. The reactions were stopped by adding SDS-PAGE loading buffer, and samples were separated by SDS-PAGE. Bands of interest were analyzed by gel trypsin digestion and mass spectrometric fingerprinting.

**Generation of HEK293 Flp-In™ T-REx™ cell lines and immunoprecipitation of complexes.** pcDNA5-based constructs for the expression of C-terminally His6-tagged ASCC2 and ASCC3 were transfected into HEK293 Flp-In™ T-REx™ cells (Invitrogen) according to the manufacturer’s instructions. Cells in which the transgene had been genomically integrated into a specific locus were selected using hygromycin and blasticidin, and population cell lines were generated. Cells were grown in Dulbecco’s modified Eagle Medium supplemented with 10% fetal bovine serum at 37°C with 5% CO2, and expression of the tagged proteins was induced by addition of 1 µg/ml tetracycline for 24 h before harvesting.

For immunoprecipitation of complexes containing Flp-tagged proteins,54–56 cells expressing full-length or truncated ASCC3-Flag or the Flag tag alone were lysed by sonication in 50 mM TRIS-HCl, pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 0.1% (v/v) Triton-X-100, 10% (v/v) glycerol and cComplete™ protease inhibitors (Roche). Cell debris were pelleted by centrifugation and the cleared lysate was incubated with anti-FlagM2 magnetic beads (Sigma-Aldrich) for 2 h. After thorough washing steps, complexes were eluted using 3xFlag peptide. Proteins were precipitated using 20% (w/v) trichloroacetic acid before separation by SDS-PAGE. Western blotting was performed using antibodies against ASCC2 (Proteintech, 11529-1-AP, diluted 1:1000) and the Flag tag (Sigma-Aldrich, F3165; diluted 1:7500). ASCC2 and ASCC3 bands were quantified using Image J software. The amount of ASCC2 relative to each ASCC3 fragment was calculated and normalized to the relative amount of ASCC2 co-precipitated with ASCC3CFL (set at 1).

**Stopped-flow/fluorescence-based helicase assays.** DNA or RNA unwinding was monitored by fluorescence-based stopped-flow on a SX20MV spectrometer (Applied photophysics). The DNA and RNA substrates contained a 12-base pair duplex region and a 31-nucleotide 3’-overhang (for sequences see Supplementary Table 4). They harbored an Alexa Fluor 488 moiety on the 3’-end of the short strand and an Atto 540 Q quencher on the 5’-end of the complementary strand, which were in close proximity after annealing. For negative control, we used a 5’-overhang DNA duplex with reverse sequences and labels (Supplementary Table 4). Experiments were conducted at 30 °C in 40 mM TRIS-HCl, pH 7.5, 80 mM NaCl, 0.5 mM MgCl2. 250 nM protein were pre-incubated with 50 nM DNA or RNA duplex for 5 min at 30°C. 60 µl of the protein–DNA or protein–RNA mixture were rapidly mixed with 60 µl of 4 mM ATP/MgCl2, and the fluorescence signal was monitored for 20 min using a 495 nm cutoff filter (KV 495, Schott). Alexa Fluor 488 was excited at 465 nm. An increase of fluorescence was observed when the duplex was separated. Control experiments included 60 µl of protein–DNA or protein–RNA mixture mixed with buffer, and DNA or RNA duplex alone mixed with ATP/ MgCl2. Data were analyzed and plotted using Prism (GraphPad).

**Gel-based helicase assays.** The same 3’-overhang DNA as for stopped-flow/fluorescence-based unwinding assays, but lacking fluorophore and quencher moieties, was used for gel-based unwinding assays (for sequences see Supplementary Table 4). The long strand was 5’-[32P]labeled by incubating 5 µl of 20 µM oligonucleotide, 20 µl of [γ-32P]ATP (Hartmann Analytic), 3 µl 10x reaction buffer (New England Biolabs) and 2 µl T4 polynucleotide kinase (New England Biolabs) for 1 h at 37°C. The labeled oligonucleotide was further passed through a Microspin G25 column (Sigma), PCI-extracted and annealed to the short, unlabeled complementary oligonucleotide in a 1:1 molar ratio in annealing buffer (10 mM TRIS-HCl, pH 8.5). Unwinding reactions were carried out at 30°C. 150 nM purified recombinant ASCC3 variants were mixed with 12 mM DNA substrate in 40 mM TRIS-HCl, pH 7.5, 0.5 mM MgCl2, 187 mM NaCl, 3.75% glycerol, 0.75 mM DTT. After a 10 min incubation at 30°C, reactions were started by addition of 5 mM ATP/MgCl2. Aliquots were withdrawn at selected time points and reactions were quenched with 50 mM TRIS-HCl, pH 7.5, 50 mM EDTA, 0.5% (w/v) SDS. For 0 min time points, all reagents except ATP/MgCl2 were added. Samples were separated on 12% non-denaturing polyacrylamide gels. DNA bands were visualized using a phosphorimager and quantified with ImageQuanti software (Cyiva). The fraction of unwound DNA in each sample was calculated as \( \frac{I_{\text{ds}}}{I_{\text{ss}} + I_{\text{ds}}} \), in which \( I_{\text{ss}} \) is the intensity of the single-stranded DNA band and \( I_{\text{ds}} \) is the intensity of the double-stranded DNA band. The fraction unwound at 0 min was subtracted from the fraction unwound for each sample.

**Reporting summary.** Further information on research design is available in the Nature Research Life Sciences Reporting Summary linked to this article.

**Data availability** Structure factors and coordinates have been deposited in the RCSB Protein Data Bank (https://www.rcsb.org/) with accession code 6YXQ. Source data for Figs. 1a–c, 3a–c, 1 j, 5c–h, 6 and Supplementary Fig. 2 are provided. Other data are available from the corresponding author upon reasonable request. Source data are provided with this paper.

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