H4K20me0 marks post-replicative chromatin and recruits the TONSL–MMS22L DNA repair complex

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After DNA replication, chromosomal processes including DNA repair and transcription take place in the context of sister chromatids. While cell cycle regulation can guide these processes globally, mechanisms to distinguish pre- and post-replicative states locally remain unknown. Here we reveal that new histones incorporated during DNA replication provide a signature of post-replicative chromatin, read by the human TONSL–MMS22L1–4 homologous recombination complex. We identify the TONSL ankyrin repeat domain (ARD) as a reader of histone H4 tails unmethylated at K20 (H4K20me0), which are specific to new histones incorporated during DNA replication and mark post-replicative chromatin until the G2/M phase of the cell cycle. Accordingly, TONSL–MMS22L binds new histones H3–H4 both before and after incorporation into nucleosomes, remaining on replicated chromatin until late G2/M. H4K20me0 recognition is required for TONSL–MMS22L binding to chromatin and accumulation at challenged replication forks and DNA lesions. Consequently, TONSL ARD mutants are toxic, compromising genome stability, cell viability and resistance to replication stress. Together, these data reveal a histone-reader-based mechanism for recognizing the post-replicative state, offering a new angle to understand DNA repair with the potential for targeted cancer therapy.

The TONSL–MMS22L complex is an obligate heterodimer required for replication fork stability and repair of replication-associated DNA damage by aiding RAD51 loading1,2. TONSL–MMS22L associates with soluble non-nucleosomal histones H3–H4 (refs 1, 5), the histone chaperone ASF1 (refs 1–4) and MCM2/4/6/7 (refs 1–5) in a manner that depends on the TONSL ARD5. We have found that histones H3–H4 bridge the interactions between TONSL–MMS22L and ASF1 (ref. 1), between ASF1 and MCM2 (refs 6, 7), and between TONSL–MMS22L and MCM2 (Extended Data Fig. 1a), suggesting simultaneous binding of these proteins to histones H3–H4 in a large pre-deposition complex. In addition, TONSL–MMS22L interacts with nucleosomal histones in chromatin (Extended Data Fig. 1b). This suggests that TONSL–MMS22L functions as an H3–H4 histone chaperone while also acting as a histone reader in chromatin. Consistent with a chaperone function, TONSL was recently shown to have histone chaperone activity in vitro3. We therefore set out to explore the mechanism of action of TONSL–MMS22L by a structure–function approach.

Full-length TONSL and the ARD alone bind directly to histones H3–H4 but not H2A–H2B (Extended Data Fig. 1c–f). As our attempts to crystallize the ARD with H3–H4 were not successful, we verified that the ARD to the MCM2 histone-binding domain (HB), because a similar design previously enabled us to solve the structure of an H3–H4 dimer in complex with MCM2 and ASF1 (ref. 7). We obtained crystals of covalently linked MCM2 HBD–G4–TONSL ARD in complex with H3 (57–135) and H4 that diffracted to 2.43 Å resolution, and solved the structure by molecular replacement on the basis of our structure of MCM2 HBD in complex with an H3–H4 tetramer7 (Fig. 1a, b; for X-ray statistics, see Extended Data Table 1). The structure shows a pair of MCM2 HBDS wrapped around the lateral surface of the H3–H4 tetramer, similar to the MCM2–HBD–H3–H4 complex alone7,8, while two TONSL ARDs interact with each of the H4 tails (Fig. 1a, b). The G4-linker along with flanking residues formed a 19-residue-long disordered segment that could reach a distance of up to 70 Å. The distance from the observed C terminus of MCM2 HBD to the observed N terminus of TONSL ARD is only 10 Å, indicating that the covalent linkage within the MCM2–HBD–G4–TONSL-ARD cassette does not affect the structural integrity of the complex. TONSL ARD forms no intermolecular interactions with the MCM2 HBD, consistent with H3–H4 bridging the interaction of TONSL and MCM2 in cells (Extended Data Fig. 1a), and it shows only minimal contacts with the core of the H3–H4 tetramer (Fig. 1a, b). However, the TONSL ARD forms extensive contacts with a segment of the H4 tail (Fig. 1b, c and Extended Data Fig. 1g) and, consistently, it binds the histone H4 tail, but not the H3 tail, in vitro (Extended Data Fig. 2a). In addition to defining TONSL binding to soluble histone H3–H4 in complex with MCM2 (Fig. 1a, b), this binding mode is also compatible with TONSL binding histone H3–H4 dimers in a co-chaperone complex with MCM2 and ASF1 (ref. 7) as well as recognizing H4 tails in a nucleosome (see models in Extended Data Fig. 2b, c).

The TONSL ARD consists of four ankyrin repeats, three of which adopt the canonical fold (ANK1–3), while the remaining one is an atypical and capping repeat (ANK4) (Extended Data Fig. 1g). The TONSL ARD uses its elongated concave surface to form extensive contacts with the H4 tail in an extended β-strand-like conformation (Fig. 1c and Extended Data Fig. 1g). Notably, 15 out of 18 residues that constitute the H4 tail-binding surface of TONSL ARD are highly conserved (Extended Data Fig. 2d). The TONSL ARD targets the H4 tail, spanning residues Lys12 to Arg23 (Fig. 1c–g and Extended Data Fig. 3a, b) with three consecutive binding channels accommodating Arg17, His18 and Lys20 (Fig. 1d). These H4 residues are part of a basic region, which can interact with the acidic patch on neighbour nucleosomes9 in compact chromatin. H4 Arg17 forms two hydrogen bonds with ARD Asn571 and stacks with Tyr572 and Cys608 (Fig. 1c, e), while H4 His18 penetrates into a pocket lined by four strictly conserved residues (Trp563, Glu568, Asn571 and Asp604) (Fig. 1c, f). Substitution of H4 His18 with the larger Trp residue (mutant H18W) disrupts...
binding with ARD (Fig. 2a), underscoring the importance of fitting His18 in the pocket. The H4 Lys20 residue is bound within an acidic surface channel on ARD (Fig. 1c, d). The side chain of H4 Lys20 interacts with Met528 and contacts the edge of Trp563 of ARD, while the main-chain atoms of H4 Lys20 packs against Cys561 of ARD (Fig. 1g). The Nε atom of H4 Lys20 forms three strong hydrogen bonds (distance <3 Å) with the side chains of strictly conserved residues Glu530, Asp559 and Glu568 of ARD, which engage H4 Lys20 in a triangular arrangement (Fig. 1g). Consistent with the structural data, histone H4 mutations R17A, H18A and K20A disrupted binding to TONSL in cells (Fig. 1h). Likewise, mutation of six conserved TONSL residues lining the H4 Arg17, His18 and Lys20 binding channels disrupted binding to H4 peptides and recombinant histone H3–H4 (Fig. 1i and Extended Data Fig. 3c). In vivo, these mutants abrogated binding to soluble histone H3–H4 and, consequently, also association with ASF1a and ASF1b and MCM2 without affecting MMS22L binding to TONSL1,2 (Fig. 1j and Extended Data Fig. 3d). These mutations did not affect ARD structure, as indicated by circular dichroism (Extended Data Fig. 3e).

![Figure 1](https://example.com/figure1.png)

Figure 1 | TONSL ARD interacts with the histone H4 tail. a, b, Two different representative views of the overall structure of the TONSL-ARD–MCM2-HBD–H3–H4 tetramer complex. c, Intermolecular interactions between TONSL ARD and the H4 tail. d, The electrostatic potential surface of ARD showing the acidic concave surface binding site for the H4 tail. e–g, Highlights of the intermolecular interactions of H4 Arg17, His18 and Lys20 with ARD. h, Immunoprecipitation (IP) of soluble haemagglutinin (HA)–SNAP–H4 wild type (WT) or mutant transfected into green fluorescent protein (GFP)–TONSL U-2-OS cells. i, ITC of TONSL ARD wild type and mutants with H4 tail peptide. j, Immunoprecipitation of soluble GFP–TONSL wild type or mutant.

h, j, Data are representative of three independent experiments. For protein inputs, see Extended Data Fig. 9b, c; for gel source data, see Supplementary Fig. 1.

![Figure 2](https://example.com/figure2.png)

Figure 2 | TONSL ARD recognizes unmodified H4K20. a, ITC of TONSL ARD binding to H4 tail peptides. b, c, Pull-down of recombinant TONSL ARD (b) or GFP–TONSL from extracts (c) with biotinylated H4 tail peptides. d, Top, pull-down of recombinant TONSL–MMS22L with biotinylated recombinant mononucleosomes. Asterisk indicates unspecific band. Unmod., unmodified. Bottom, TONSL binding quantified relative to histones. Unpaired t-test: *P < 0.05; mean n = 6; whiskers, outliers. e, As in c, f, Immunoprecipitation of GFP–TONSL from solubilized chromatin. g, TONSL chromatin binding in pre-extracted SET8-depleted G1 cells. AU, arbitrary units. Error bars indicate standard deviation (s.d.); n = 747 (short interfering RNA (siRNA) control), 579 (SET8#1) and 485 (SET8#2). Data are representative of four (b), three (c) and two (e–g) independent experiments. For peptide inputs, see Extended Data Fig. 9d.
bars indicate s.d.; relative to total GFP–TONSL and normalized to wild type (WT). Error binding of GFP–TONSL analysed by cellular fractionation, quantified in independent experiments.

Furthermore, H4K20me2 significantly reduced binding of full-length monomeric TONSL–MMS22L to reconstituted mononucleosomes (Fig. 2d). Recently, TONSL ARD with its neighbouring acidic stretch was proposed to bind H3K9me1 (ref. 5), but we were unable to detect an interaction between TONSL ARD (with or without the acidic stretch) and H3K9me1 peptides (Extended Data Fig. 4c, d). Together, our data show that TONSL binds to both free histones and nucleosomes via ARD recognition of H4 tails unmodified at K20 (Figs 1a–j, 2a–e and Extended Data Figs 1b, 2a–c, 3a–d). In line with this, H4K20me2 was not detected on TONSL-bound nucleosomal histones (Fig. 2f), while H4K16ac was present (Extended Data Fig. 5a). H4K16ac stimulated TONSL binding in peptide pull-downs (Fig. 2b, c and Extended Data Fig. 5b) and slightly enhanced ARD binding in ITC (Fig. 2a), but it did not overturn the inhibitory effect of H4K20me2 (Fig. 2e). However, H4K16ac is not essential for TONSL binding in vivo, as soluble histone H4 does not carry H4K16ac11 and depletion of MOF, the major H4K16 acetyltransferase12, did not significantly affect TONSL binding to chromatin (Extended Data Fig. 5c, d).

Given that TONSL–MMS22L binds new histones (devoid of H4K20me1,17) in a pre-deposition complex with asf1 and MCM2 (Fig. 1j and Extended Data Fig. 1a), TONSL–MMS22L could be loaded onto replicating DNA together with new histones. To test how long after deposition new histones remain unmethylated at H4K20 with the potential to bind TONSL, we extracted H4K20 data from our recent large-scale proteomic study14, tracking modifications on new and old recycled histones by nascent chromatin capture (NCC)19 (Fig. 3a and Extended Data Fig. 6a, b). In nascent chromatin, new histones were exclusively unmethylated at H4K20 (98% H4K20me0), while old recycled histones were almost fully methylated at H4K20 (me1, 7%; me2, 88%; me3, 2%). Consistent with previous work13–16, our analysis of primary cells (Extended Data Fig. 6c) and degradation of set8 in S phase15,16, new histones became methylated in late G2/M, rendering G1 chromatin devoid of H4K20me0 (Fig. 3a). This identifies H4K20me0 on new histones as a signature of post-replicative chromatin, implying that TONSL–MMS22L can bind H4 tails on new histones at replication forks and sister chromatids until late G2/M. Confirming this prediction, TONSL accumulated on chromatin in S phase, remained chromatin-bound in a population of G2 cells, and was excluded from chromatin in G1 (Fig. 3b and Extended Data Fig. 6d–f). To discriminate pre- and post-replicative chromatin, we labelled replicating DNA with 5-ethylideneoxyuridine (EdU; pulse to mark ongoing replication, continuous labelling to identify post-replicative chromatin) and stained pre-replicative chromatin with MCM2 (refs 20, 21), and analysed colocalization with TONSL. TONSL staining was mutually exclusive with MCM2 (Fig. 3c and Extended Data Fig. 7a), but colocalized with EdU pulse labelling in very early S phase and with replicated DNA (continuous EdU labelling) throughout S phase (Fig. 3d and Extended Data Fig. 7b, c). TONSL was present at sites of ongoing DNA replication throughout S phase, but the degree of colocalization declined in mid/late S phase (Fig. 3d, left), consistent with TONSL binding to post-replicative chromatin also after fork passage (Fig. 3d, right). Mutation of the TONSL ARD abrogated recruitment of TONSL to chromatin, including DNA replication sites (Fig. 3e, f and Extended Data Fig. 7d–g). Together, these data demonstrate that TONSL is recruited to replication forks and post-replicative chromatin via ARD recognition of H4K20me0 on new histones.

Mutation of the TONSL ARD also abrogated chromatin binding and recruitment to replication forks in the presence of replication poisons such as camptothecin (CPT) and hydroxyurea (Fig. 4a–c). Furthermore, ARD mutation prevented accumulation of TONSL at site-specific double-strand breaks (DSBs; Fig. 4d and Extended Data Fig. 8a) and microcell-generated DNA damage (Fig. 4e and Extended Data Fig. 8b, c). Co-staining with cell cycle markers confirmed that

Together, this defines TONSL ARD as a recognition module for histone H4 tails, distinct from the GLP/G9A ARDs that bind histone H3 tails mono- or dimethylated at K9 (Extended Data Fig. 4a, b)10.

The structure predicts that methylation on H4K20 should break critical hydrogen bonds with the TONSL ARD. Isothermal titration calorimetry (ITC) and H4-tail peptide pull-downs confirmed that H4K20me1 is incompatible with TONSL binding (Fig. 2a–c). Furthermore, H4K20me2 significantly reduced binding of full-length recombinant TONSL–MMS22L to reconstituted mononucleosomes.
H4K20me0 recognition is required for TONSL accumulation at DNA repair sites and genome stability. a, Chromatin-binding of GFP–TONSL in CPT-treated S-phase cells. WT, wild type. Error bars indicate s.d.; from left, n = 1,461, 2,631, 1,245, 1,764, 2,116, 3,178. b, Co-immunoprecipitation (IP) of TONSL–MMS22L with Flag–HA–MCM2 wild type or histone-binding mutant (Y81A, Y90A)2 from chromatin after hydroxyurea (HU) treatment. c, NCC analysis of GFP–TONSL recruitment to replication forks in CPT-treated cells. Minus sign indicates no b-dUTP. d, Chromatin immunoprecipitation (ChIP) and quantitative polymerase chain reaction (qPCR) analysis of GFP–TONSL recruitment to site-specific DSBs induced by AsiSI29. See Extended Data Fig. 8a for additional controls. e, GFP–TONSL recruitment to laser-induced DNA lesions (error bars indicate s.d.; n = 3; total cells counted, 210 (wild type) and 252 (N571A)). f, g, Colony formation upon GFP–TONSL induction by tetracycline (+tet) in siRNA- and CPT-treated cells. h, Cell cycle and 53BP1 foci analysed by microscopy. Left, percentage of G2/M cells shown relative to non-induced cells (−tet). Error bars indicate s.d., n = 4 (left), 5 (right). i, Chromatin-bound MMS22L analysed as in Fig. 3e. Mean with individual data points are shown (n = 3 (untreated), 2 (CPT)), see Extended Data Fig. 8i for western blots. j, TONSL–MMS22L identifies post-replicative chromatin by binding H4K20me0 on new histones, directing TONSL–MMS22L genome surveillance function to DNA having a sister chromatid. Data are representative of three (a), two (b–d, f, right, g), and four (f, left) independent experiments. For protein inputs, see Extended Data Fig. 9e, f.

TONSL is recruited to DNA repair sites only in S and G2 cells, as expected2 (Fig. 4e and Extended Data Fig. 8d, e). We conclude that H4K20me0 binding is required for TONSL accumulation at damaged forks and DNA lesions in post-replicative chromatin. However, this was not due to increased H4K20me0 (Extended Data Fig. 8f), suggesting that unmasking of H4 tails upon chromatin decompaction17,22 and/or interaction with repair factors contribute to TONSL–MMS22L accumulation at repair sites. Consistent with an auxiliary mode of recruitment, MMS22L interaction with RAD51 can stabilize the complex at challenged forks (P. Cejka and M. Peter, personal communication). Our data suggest that this is subsequent to H4K20me0 recognition (Fig. 4a–e), and we thus next addressed the contribution of H4K20me0 recognition to TONSL–MMS22L function. In complementation analysis, TONSL wild type partially rescued the viability of TONSL-depleted cells in the presence and absence of CPT (Fig. 4f and Extended Data Fig. 8g, h), whereas TONSL ARD mutants were toxic (Fig. 4f and Extended Data Fig. 8g, h). In control cells, TONSL ARD mutants also reduced viability, causing G2/M arrest accompanied by replication-associated DNA damage (Fig. 4g, h). Furthermore, TONSL ARD mutants titrated MMS22L away from chromatin (Fig. 4i and Extended Data Fig. 8i), explaining the dominant-negative phenotype that mimics TONSL–MMS22L depletion14. Collectively, this indicates that recognition of H4K20me0 is central to TONSL–MMS22L function in safeguarding genome stability.

This study reveals that post-replicative chromatin has a distinct histone modification signature, read by the TONSL–MMS22L effector protein (Fig. 4j). This opens a new avenue to understand how DNA repair and other chromosomal transactions can be directly linked to the replication state of a genomic locus. Intriguingly, it is the new histones that make post-replicative chromatin distinct, and in this way H4K20me0 resembles the behaviour of H3K56ac23 in yeast. Our data indicate that TONSL–MMS22L is delivered to nascent chromatin with post-replicative chromatin by binding H4K20me0 on new histones, directing TONSL–MMS22L genome surveillance function to DNA having a sister chromatid. Data are representative of three (a), two (b–d, f, right, g), and four (f, left) independent experiments. For protein inputs, see Extended Data Fig. 9e, f.
lesions. We envision that H4K20me0 works as an apoptosis trap, making TNSL1–MMS21L readily available to support RAD51 loading during homologous recombination. This provides a new approach and opportunity to understand the role of H4K20 in DNA repair, complementing the well-described role of H4K20me1 in recruiting 53BP1 to promote non-homologous end joining in competition with BRCA1–BARD1 (refs 24, 25). In post-replicative chromatin, H4K20me1/2 on old histones will support 53BP1 recruitment. Whether H4K20me0 on new histones also influences DNA repair pathway choice will be of interest in future investigations. It is notable that the structure of the TONS1 ARD, including the histone-binding surface, is highly similar to the ARD of BARD1 (Extended Data Fig. 9a)26, required for BRCA1 tumour suppressor function and homologous recombination27. Multiple mutations in the TONS1 ARD are reported in cancer (C608G, P557S, E597K; http://cancer.sanger.ac.uk) and the N571 residue, key to histone H4 binding, corresponds to the BARD1 N470S cancer mutation26,28. This highlights the tumour suppressor function of H4K20me0 recognition, and the possibilities it brings for targeted cancer therapy should be explored in the future.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions G.S. and A.G. conceived and led the functional studies. H.H conceived and led the generation of cassette to crystalize the complex, and H.H. solved the structure and performed the ITC under the supervision of D.J.P. C.M.H. performed peptide pull-downs with ARD and recombinant nucleosomes. C.A. performed SET8 experiments and NCC. S.B.-J. and N.M. analysed recruitment to laser-induced DNA damage. N.R.-G. prepared histones for mass spectrometry and performed ChIP analysis, I.F. analysed histone modifications by mass spectrometry under the supervision of A.I. B.M.F. and T.B. prepared modified recombinant nucleosomes. L.M. and P.C. performed combinatorial TNSL1–MMS21L. G.S., H.H., D.J.P. and A.G. wrote the manuscript and all authors commented on the manuscript.

Author Information Coordinate and structure factors have been deposited in the Protein Data Bank under accession number 5JAU. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to D.J.P. (pateld@mskcc.org) or A.G. (anja.groth@bric.ku.dk).

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METHODS

Protein expression and purification. No statistical methods were used to pre- determine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment. All proteins used in this study, unless otherwise indicated, were expressed in BL21(DE3)-RIL cell strain (Stratagene). The human TONS1 ARD (residues 512–692) and MCM2 HBD (fragments 61–130) were covalently linked through a four-glycine linker (G4 linker) into one expression cassette. The MCM2 HBD–G4–TONSL ARD expression cassette was cloned into a modified RSFDuet-1 vector (Novagen), with an N-terminal His6–SUMO tag. The resulting plasmid was co-expressed with plasmid harbouring histone genes H3.3(17–236) and H4. The expressed protein complex was first purified on an Ni-NTA affinity column. After removing the His6–SUMO tag by using Ulp1 (SUMO protease), the protein complex was further purified on HiLoad 16/600 Superdex 200 column (GE Healthcare).

The GST-tagged TONS1 ARD and its mutants including E530A, D559A, W563A, E568A, N571A and D640A were cloned into pGEX-6P-1 vector (GE Healthcare). The expressed proteins were first purified using Glutathione Sepharose 4B, then further purified by gel-filtration step. In some case, the GST tag was removed with 3C protease before the gel-filtration step. For purification of GST–H3 tail and GST–H4 tail proteins, the human histones H3 fragment 1–59 and H4 fragment 1–31 were cloned into pGEX-6P-1 vector respectively. The proteins were expressed and purified in the same way.

For production of recombinant full-length TONS1–MMS22L heterodimer, the sequence coding for full-length MMS22L was fused with an MBP tag at the 5′ end and 10× His tag at the 3′ end. The sequence coding for full-length TONS1 was fused with a GST tag at the 5′ end. Both MMS22L and TONS1 constructs were cloned into a pFastBac1 vector. The complex was expressed in S9 cells by co-infection with both recombinant baculoviruses according to manufacturer’s recombinant baculovirus expression system (Invitrogen). The proteins were extracted from S9 cells and purified similarly as described previously for Sgs1 (ref. 30). Briefly, the complex was purified on amylose resin, and MBP and GST tags were subsequently cleaved with PreScission protease. The heterodimeric complex was then further purified using a Ni-NTA affinity resin. Washes were performed with 300 mM NaCl buffer.

Crystalization. At first, we tried to crystallize TONS1 ARD in complex with a H3 tail or H4 tetramer, but failed even with extensive screening. An additional binding protein may help to stabilize the whole complex and help crystallization. Then we tried to crystallize TONS1 ARD in complex with the MCM2 HBD and H3–H4 tetramer. We just got very tiny crystals for this complex, but failed to get purified on amylose resin, and MBP and GST tags were subsequently cleaved with PreScission protease. The heterodimer was then further purified using a Ni-NTA affinity resin. Washes were performed with 300 mM NaCl buffer.

Structure determination. The data sets for the TONS1–ARD–MCM2-HBD–H3–H4 tetramer complex was collected at a concentration of 23 mg ml\(^{-1}\) crystallized in 0.1 M MES pH 5.6, 7% iso-propanol using sitting-drop vapour-diffusion method at 20 °C. All the crystals were soaked in a cryoprotectant made from mother liquor supplemented with 7% isopropanol at 20 °C. All the crystals were processed with Microcal Origin software and the curves were fit to a single site binding model. The data were processed and reduced using HKL 2000 and SCALEPACK software packages.

Preparation of recombinant modified mononucleosomes. Recombinant human histone proteins were expressed in Escherichia coli BL21(DE3)-RIL cells from pET21b(+) (Novagen) vectors and purified by denaturing gel filtration and ion-exchange chromatography essentially as described31. All histone proteins were dialysed into water containing 1 mM dithiothreitol (DTT), lyophilized and stored dry at –80 °C. Modified H4 proteins were generated by native chemical ligation essentially as described for H3 (ref. 35). Briefly, tail-less H3–L2 was labelled at Lys20, and peptide of H3(1–21)K9me1 (monomethylation on Lys9) were all synthesized at Tufts University Core Facility. All the data sets were processed with Microcal Origin software and the curves were fit to a single site binding model. For pull-downs from cell extracts, MyOne T1 beads were incubated overnight with 2 μg 35S-Met and 35S-cysteine in 1 ml binding buffer (20 mM Tris pH 7.5 and 0.5 M NaCl), and 1 nmol of GST–ARD proteins were added and incubated at 23 °C for 10 min; then 0.5 nmol of pre-purified H3/H4 tetramers were added and incubated for another 1 h; then the beads were washed quickly with five times 1 ml of washing buffer (binding buffer, 1% Triton X-100) before adding 50 μl of sample loading buffer. An aliquot of 20 μl of each sample was analysed with SDS–PAGE. The GST pull-downs of histone tails of GST–H3.3 and GST–H4.31 with TONS1 ARD were performed similarly.

Circular dichroism. Circular dichroism spectra were acquired using a Jasco J-815 Circular Dichroism Spectropolarimeter with a 1 mm quartz cuvette. Spectra were recorded for wild-type and mutant TONS1 ARD (512–692, 6.25μM) between 260 nm and 195 nm in HK2P04/K2HPO4 buffer (25 mM, pH 7.8) with a data pitch of 0.5 nm, bandwidth of 1 nm and with three accumulations at a scanning speed of 50 nm min\(^{-1}\).

In vitro translation and pull-downs with H3–H4 sepharose beads. NHS-activated sepharose 4 Fast-Flow beads (GE Healthcare) were washed with 0.1 M HCl and incubated overnight with 1 μM recombinant histone H3.1–H4 tetramers (New England Biolabs, catalogue number M2509S) or 1 μM recombinant histone H2A–H2B dimers (New England Biolabs, catalogue number M2508S) in Coupling buffer (0.2 M NaHCO\(_3\), 0.2 M NaCl). One microgram of pSc8-B-TONS1, pEXPRIBA-105-AF1A-V94R plasmids was incubated with Tnt Quick Coupled Transcription/Translation System (Promega) and \(^{35}\)S-methionine according to the manufacturer’s instructions. Ten microlitres of in vitro translation (IVT) mixture were added to the H3.1–H4, or H2A–H2B sepharose beads and incubated for 2 h. Beads were washed with 200 mM NaCl, 0.2% NP40 buffer. Beads were boiled in 1× LSB and loaded on a 4–12% Bis-Tris NuPage gel (LifeTechnologies). Proteins were transferred to a 0.2 μm nitrocellulose membrane by overnight wet transfer at 20 V and the membrane was incubated in an autoradiography cassette for 24 h before detection by Phosphor Imager (PerkinElmer).

ITC experiments. All the ITC titrations were performed on a Microcal ITC 200 calorimeter at 25 °C or 20 °C. The peptides of H4 (residues 9–25) and its modified peptides K16ac (with acetylation on Lys16), H18W (with His18 mutated to Trp18), H4K20me1 (monomethylation on Lys20) and H4K20me2 (dimethylation on Lys20), and peptide of H3(1–21)K9me1 (monomethylation on Lys9) were all synthesized at Tufts University Core Facility. The exothermic heat of the reaction was measured by 17 sequential 2.2 μl injections of the peptides (1.41 mM in buffer 20 mM Tris pH 7.5 and 0.5 M NaCl) into 200 μl of the TONS1 ARD solution (145 μM in the same buffer), spaced at intervals of 150 or 180 s. The data were processed and reduced using Microcal Origin software and the curves were fit to a single site binding model. The peptide pull-downs assays. Purified recombinant TONS1 ARD (residues 512–692) was stored at 400 μM in 1 M NaCl, 20 mM Tris HCl pH 7.5 at ~80 °C. For each pull-down, 400 pmol of the ARD stock (1 μl, 400 μM) was diluted with 99 μl of binding buffer (150 mM NaCl, 50 mM Tris HCl pH 7.5, 5% glycerol, 0.25% NP40, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSE 1 mM leupeptin, 1 mM pepstatin). ARD input material was scaled to the number of pull-downs performed. For each pull-down, an H4 peptide (IPTP Peptide Technologies GmbH) spanning residues 14–33 (2.5 μl, 250 μM) with a C-terminal biotinoyl-lysine residue or, as control, biotinylated H4 peptide of H4(9–25) was added to the beads and incubated in addition to 100 μl of the ARD input material and the mixture was incubated overnight rotating at 4 °C. The next day, 25 μl of MyOne Streptavidin C1 beads (Life Technologies) was washed in binding buffer (3 × 500 μl) for each pull-down, removing the final wash from the beads. The ARD/peptide or ARD/biotin mixture was added to an aliquot of pre-washed MyOne Streptavidin C1 beads and incubated with rotation at 4 °C for 5 h. Finally, the beads were washed (2 × 300 μl and 1 × 200 μl of 300 mM NaCl, 50 mM Tris HCl pH 7.5, 5% glycerol, 0.25% NP40, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSE 1 mM leupeptin, 1 mM pepstatin) and pull-down material was visualized by Coomassie staining after SDS–PAGE separation of proteins on a 10% SDSPAGE gel.
then washed five times with H5 buffer, 2 min rotating at 4 °C. After washing, the beads were resuspended in 1× LSB and boiled for 10 min. The eluted proteins were loaded on a 4–12% Bis-Tris NuPage gel (Life Technologies). Proteins were then transferred to a 0.2 μm nitricellulose membrane by overnight wet transfer at 20 V and detected by western blotting.

**Nucleosome pull-down assay.** Modified nucleosomes of H4K20me0 or H4K20me2 were prepared by peptide ligation and stored at 0.1 μg μl−1 (by histone octamer) in 100 mM NaCl, 50 mM Tris HCl pH 7.5 at 4 °C. Full-length TONSIL–MMS22L1 (1.9 pmol) and made up to 30 μl with binding buffer (500 μM NaCl, 50 mM Tris HCl pH 7.5, 20% glycerol, 0.1% NP-40, 1 mM DTT, protease inhibitors and 10 μg ml−1 herring sperm DNA (Sigma)). Inputs of 10 μl were taken before diluting each sample with binding buffer to a final volume of 300 μl and incubating overnight at 4 °C. Pull-downs were performed by adding 20 μl of MyOne Streptavidin C1 beads preswollen and resuspended in 100 μl of binding buffer to each pull-down reaction, incubating at 4 °C for 2 h, washing with 5× 500 μl binding buffer for 2 min at room temperature. Condition number 2: nucleosomes (0.5 μg by histone octamer) or biotin (0.5 μg) were mixed with TONSIL–MMS22L1 (1.3 pmol) and made up to 30 μl with binding buffer (500 μM NaCl, 50 mM Tris HCl pH 7.5, 5% glycerol, 0.5% NP-40, 0.2 mM EDTA, 1 mM DTT, protease inhibitors and 10 μg ml−1 herring sperm DNA (Sigma)). Inputs of 15 μl were taken before diluting each sample with binding buffer to a final volume of 500 μl and incubating overnight at 4 °C. Pull-downs were performed by adding 10 μl of MyOne Streptavidin T1 beads preswollen and resuspended in 100 μl of binding buffer to each pull-down reaction, incubating at 4 °C for 4 h, washing with 5× 500 μl binding buffer for 2 min at room temperature. Pull-downs were visualized by SYPRO Ruby staining after SDS–PAGE separation of proteins on a NuPAGE 4–12% gel using an ImageQuant LAS 4000 (GE Healthcare). The intensity of stained bands were quantified using ImageJ. TONSIL intensity was normalized to the combined intensity of H3, H2A and H2B. Statistical analysis was performed using data from the six independent experiments using the unpaired t-test with equal standard deviations in prism 6.

**Cell culture, transfection and drug treatment.** U-2-OS (gift from J. Bartek), HeLa S3 (gift from P. Nakatani) and TIG-3 (gift from K. Hansen) cells were grown in DMEM (Gibco) containing 10% FBS (Hyclone) and 1% penicillin/streptomycin and drugs for selection. The construct for pBABE-SNAP-HA-H4 plasmid was prepared by peptide ligation and stored at 0.1 μg μl−1 (by histone octamer) or biotin (0.5 μg) by histone octamer) or biotin (0.5 μg) and subsequently centrifuged at 1,500 rpm for 10 min. The protein was solubilized in 5 mM β-mercaptoethanol, 10% glycerol, 0.5 mM PMSF at 80 °C. Nucleosome pull-downs were performed across two sets of conditions (n = 3 for each condition) in the presence of herring sperm competitor DNA. Condition number 1: Nucleosomes (1 μg by histone octamer) or biotin (0.5 μg) were mixed with TONSIL–MMS22L1 (1.9 pmol) and made up to 30 μl with binding buffer (500 μM NaCl, 50 mM Tris HCl pH 7.5, 20% glycerol, 0.1% NP-40, 1 mM DTT, protease inhibitors and 10 μg ml−1 herring sperm DNA (Sigma)). Inputs of 10 μl were taken before diluting each sample with binding buffer to a final volume of 300 μl and incubating overnight at 4 °C. Pull-downs were performed by adding 20 μl of MyOne Streptavidin C1 beads preswollen and resuspended in 100 μl of binding buffer to each pull-down reaction, incubating at 4 °C for 2 h, washing with 5× 500 μl binding buffer for 2 min at room temperature. Condition number 2: nucleosomes (0.5 μg by histone octamer) or biotin (0.5 μg) were mixed with TONSIL–MMS22L1 (1.3 pmol) and made up to 30 μl with binding buffer (500 μM NaCl, 50 mM Tris HCl pH 7.5, 5% glycerol, 0.5% NP-40, 0.2 mM EDTA, 1 mM DTT, protease inhibitors and 10 μg ml−1 herring sperm DNA (Sigma)). Inputs of 15 μl were taken before diluting each sample with binding buffer to a final volume of 500 μl and incubating overnight at 4 °C. Pull-downs were performed by adding 10 μl of MyOne Streptavidin T1 beads preswollen and resuspended in 100 μl of binding buffer to each pull-down reaction, incubating at 4 °C for 4 h, washing with 5× 500 μl binding buffer for 2 min at room temperature. Pull-downs were visualized by SYPRO Ruby staining after SDS–PAGE separation of proteins on a NuPAGE 4–12% gel using an ImageQuant LAS 4000 (GE Healthcare). The intensity of stained bands were quantified using ImageJ. TONSIL intensity was normalized to the combined intensity of H3, H2A and H2B. Statistical analysis was performed using data from the six independent experiments using the unpaired t-test with equal standard deviations in prism 6.

**Western blotting and antibodies.** The following antibodies were used: TONSIL (Abcam ab101898), TONSIL (Sigma, HPA024679; validated in Extended Data Fig. 6d), MMS22L1, H3 (Abcam ab1791, Abcam ab10799), GFP (Santa Cruz sc-8334, Abcam ab290), biotin (Abcam ab34949), MCM2 (BD Biosciences 610701), H2B (Abcam ab1790), H4K16ac (Millipore 07-329), H4K20me1 (Abcam ab9051), H4K20me2 (Cell Signaling 9759), 53BP1 (Santa Cruz sc-22760; Novus Biologicals NB100-904), γ-H2AX (Millipore 05-636), Cyclin B (BD Biosciences 610220), RPA70 (Abcam ab79398), SETR8 (Millipore, 06-1304), MCM3 (Abcam ab 4460). Secondary antibodies conjugated with horseradish peroxidase (HRP) were from Jackson ImmunosResearch Labs. Signals were revealed by chemiluminescence substrate from Pierce (SuperSignal West Pico or SuperSignal West Femto).

**FACS and analysis.** For analysis of cell cycle progression, cells were fixed in 70% ethanol and stained with propidium iodide/RNase for 30 min in the dark, before analysis on a FACS Calibur machine. FACS profiles were analysed by FlowJo 10.0.8 software.

**Mass spectrometry.** Histones from TIG3 fibroblasts were extracted from chromatin as previously described14. Protein was resuspended in 50 μl of 100 mM triethylammonium bicarbonate (TEAB, Sigma), pH adjusted with 2 μl of 1.5 M Tris pH 8 and digested for 16 h at 37 °C with 3 μl of 20 ng μl−1 Asp-N (Wako) in 100 mM TEAB. After 15 min centrifugation at 10,000 g at 25 °C, the supernatant was placed in a new tube and digestion was repeated for the pellet during 4 h under the conditions described earlier. The digested peptides of both digestions were mixed, acidified with 10 μl of 1% TFA and purified using sequential Stagetip C18 and Carbon Top tip (Glygen). Purified peptides were evaporated, resuspended in 15 μl of 0.1% TFA. Injected material was normalized to analyse by liquid chromatography mass spectrometry (LC-MS) the histones corresponding to 9.0 × 109 cells. The LC method was used as described elsewhere15. The MS was performed in an Orbitrap Classic with similar settings as described previously15 but with survey scan range of 280–690 m/z and MS2 set in scheduled and targeted data dependent mode for the four-time charged ions of the four different methylation states (unmodified, mono-, di- and trimethylated H4K20). Peptides were identified using the peak area from the corresponding extracted ion chromatograms (± 10 p.p.m.).

**Immunofluorescence, microscopy and laser microirradiation.** U-2-OS cells conditional for GFP–TONSL were grown on glass coverslips or 96-well plates and either directly fixed in 4% paraformaldehyde (PFA) for 10 min or washed in CSK, pre-extracted 5 min with cold CSK (0.5% Triton X-100 and rinsed with CSK and PBS before fixation in 4% PFA for 10 min. Coverslips were mounted on glass slides with Durotix (Bio] The microirradiation experiments, cells grown on glass coverslips were fixed in 4% formaldehyde for 15 min, permeabilized with PBS containing 0.2% Triton X-100 for 5 min and incubated with primary antibodies diluted in DMEM for 1 h at room temperature. After staining with secondary antibodies (Alexa Fluor 488, 567 and 647; Life Technologies) for 30 min, coverslips were mounted on glass slides in Vectashield mounting medium (Vector Laboratories) containing...
the nuclear stain DAPI. For detection of nucleotide incorporation during DNA replication, an EdU-Plus labelling kit (Life Technologies) was used according to the manufacturer’s instructions. Confocal images were acquired on an LSM-780 (Carl Zeiss) mounted on a Zeiss-AxioObserver Z1 equipped with a Plan-Neofluar ×40/1.3 oil immersion objective. Image acquisition and analysis was carried out with LSM-ZEN software. Laser microirradiation of cells was performed essentially as described 38.

ChIP. GFP–TONSL wild type and N571A U-2-OS harbouring the inducible ER–HA–AsiSI endonuclease 29 were treated with 4-OHT and 10 μM DNA-PK inhibitor NU7026 (Millipore) for 4 h to increase homologous recombination 39. Cells were cross-linked for 10 min in 1% formaldehyde and chromatin was fragmented by sonication using Bioruptor Sonicator (Diagenode). ChIP was performed as previously described 40 with the following modifications: 30 μg of chromatin was immunoprecipitated with 5 μg of anti-GFP (Abcam ab290) and rabbit-IgG. Immunoprecipitated DNA was analysed in duplicate by RT–qPCR. For the analysis of DSBS, DSB-I and DSB-II are described 41. Primer sequences used for the amplification of a genomic region of DSBS were as follows: noDSB-for: 5′-TGACAAGGACAGGGTCTTCC; noDSB-rev: 5′-CACCGTCCGGTTGTATGCTCTG. ChIP efficiency was calculated as percentage of input DNA immunoprecipitated.

NCC. The NCC protocol 19 was adjusted for adherent U-2-OS cells. CPT (1 μM) was added 5 min before b-dUTP labelling and was included in all steps until fixation. Cells were incubated for 5 min in a hypotonic buffer (50 mM KCl, 10 mM HEPES) containing b-dUTP and resuspended into fresh cell culture medium for an additional 15 min. Cells were fixed 15 min in 1% formaldehyde, rinsed twice in PBS and collected by scraping in cold room. Nuclei were mechanically sheared for 90 s off in sonication buffer (10 mM HEPES-NaOH at pH 7.9, 1% Triton X-100 and 2 mM MgOAc). Chromatin was solubilized by 28 cycles 30 s on, 90 s off in sonication buffer (10 mM HEPES-NaOH at pH 7.9, 100 mM NaCl, 2 mM EDTA at pH 8, 1 mM EGTA at pH 8, 0.2% SDS, 0.1% sodium sarcosyl and 1 mM phenylmethylsulfonylfluoride) using a Bioruptor at 4°C. Solubilized chromatin was pre-cleared using streptavidin-coated magnetic beads. Chromatin was next purified over night at 4°C using streptavidin-coated magnetic beads. Beads were washed five times for 2 min in wash buffer (10 mM HEPES-NaOH pH 7.9, 200 mM NaCl, 2 mM EDTA pH 8, 1 mM EGTA pH 8, 0.1% SDS, 1 mM PMSF). Total chromatin (input) and isolated nascent chromatin were boiled for 40 min on beads in LSB 1× (50 mM Tris-HCl pH 6.8, 100 mM DTT, 2% SDS, 8% glycerol, bromophenol blue) and separated by SDS–PAGE for western blotting. Pulse-SILAC-NCC (Fig. 3a) was performed as described 42.

Clonogenic assay. U-2-OS inducible for GFP–TONSL ARD wild type and mutant were transfected with siRNA, trypsinized 24 h later and seeded in technical triplicates of 1,000 or 3,000 cells in the presence or absence of tetracycline. After 24 h, cells were washed to remove tetracycline and CPT was added for 24 h as indicated. Cells were then cultured in fresh medium for 12–15 days before fixation and staining with MeOH/Crystal Violet. Colony formation efficiency was determined by manual colony counting or quantification of Crystal Violet staining by ImageJ software and normalized to non-induced control. Each data point represents a technical triplicate of 1,000 or 3,000 seeded cells within each biological replicate.

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Extended Data Figure 1 | TONSL binding to histones in vivo and in vitro. 

a, Histones bridge the interaction between TONSL–MMS22L and MCM2 in cell extracts as shown by co-immunoprecipitation of Flag–HA–MCM2 wild type or histone-binding mutant (Y81A, Y90A)7. U–2–OS cell inducible for Flag–HA–MCM2 wild type or Y81A, Y90A7 were induced for 24 h before immunoprecipitation with Flag antibodies (one representative experiment out of two is shown). 

b, Immunoprecipitation of GFP–TONSL from solubilized chromatin of HeLa cells transiently transfected with GFP–TONSL plasmid, showing that TONSL associates with nucleosomal histones H3 and H2B (one representative experiment out of two is shown).

c, Domain structure of TONSL1–4. LRR, leucine-rich repeats; TPR, tetratricopeptide repeats; UBL, ubiquitin-like domain.

d, Pull-down of GST–ARD with recombinant histones H3–H4 tetramers.

e, Pull-down of in vitro-translated full-length TONSL with recombinant histones H3–H4 tetramers (e) or H2A–H2B dimers (f) coupled to NHS-activated sepharose beads (one representative experiment out of three (e) and two (f) is shown). ASF1a wild type and histone-binding mutant (V94R) were included as controls.

g, TONSL ARD consists of four ankyrin repeats and uses its elongated concave surface to target the H4 tail spanning residues 12 to 23.
Extended Data Figure 2 | See next page for caption.
Extended Data Figure 2 | Models and sequence alignment of TONSL ARD.  

**a.** Pull-down assay of recombinant ARD with GST–H3 tail (amino acids 1–59) and GST–H4 tail (amino acids 1–31).  

**b.** Modelling of TONSL ARD on the co-chaperone structure of MCM2 HBD and ASF1 in complex with an H3–H4 dimer. When comparing the structure of the TONSL-ARD–MCM2-HBD–H3–H4 tetramer complex with our previous structure of the MCM2-HBD–H3–H4-dimer–ASF1 complex (Protein Data Bank accession 5BNX), the common parts of both structures superimposed well with a small root mean squared deviation (r.m.s.d.) of 0.44 Å. A model of the quinary complex composed of one molecule of each protein, TONSL ARD, MCM2 HBD, ASF1, H3 and H4, was made after superposition. This model shows that TONSL ARD, MCM2 HBD and ASF1 could simultaneously bind an H3–H4 dimer without steric clash.  

**c.** Model of TONSL ARD on the structure of the nucleosome. The model was generated by a direct superposition of the H3–H4 tetramer in the structure of the TONSL ARD–MCM2 HBD–H3–H4 tetramer complex onto the H3–H4 tetramer in the nucleosome structure (Protein Data Bank accession 3AV2). There was no adjustment in the conformation of the model and no steric clash in the model. The MCM2 HBD molecules were omitted from the model for clarity.  

**d.** Alignment of TONSL ARD (512–692) sequences from *Homo sapiens*, *Mus musculus*, *Xenopus laevis* and *Danio rerio*. The secondary structures of human TONSL ARD are showed on top of the sequence alignment. Asterisks indicate the highly conserved residues that constitute the H4 tail-binding surface of TONSL ARD and the three strictly conserved acidic residues forming hydrogen bonds with the key residue H4 Lys20 are highlighted with red asterisks.
Extended Data Figure 3 | Interaction details of TONSL ARD and GST pull-downs. a, b, Molecular details of the interactions of TONSL ARD with H4 tail region residues 12–15 (a) and residues 21–23 (b). The Lys12-Gly13-Gly14-Ala15 segment of H4 is positioned within a narrow surface channel of the TONSL ARD scaffold. The intermolecular contacts spanning the Lys12-Gly13-Gly14-Ala15 segment of H4 include hydrophobic interactions between residues Gly13, Gly14 and Ala15 of H4 and residues Asn507, Cys508, Trp641, Tyr645 and Leu649 of ARD, as well as hydrogen bonds between the main-chain O of H4 Gly14 and Nε1 of ARD Tyr645 (a; Fig. 1c). The main-chain O of H4 Lys16 hydrogen bonds with the Nδ2 of ARD Asn571, while the side chain of H4 Lys16 forms contacts with ARD Asn607 and electrostatic interactions with the side chain of ARD Glu597 (Fig. 1c). The side chain of H4 Arg17 stacks over the side chains of ARD Tyr572 and Cys608, while its Nη1 atom forms two hydrogen bonds with main-chain O and Oδ1 of ARD Asn571 (Fig. 1c, e). The side chain of H4 H18 penetrates into a pocket lined by four strictly conserved residues (Trp563, Glu568, Asn571 and Asp604) and is positioned over His567 of ARD (Fig. 1c, f). The side chain of H4 His18 is stacked between Trp563 and Asn571 and forms hydrogen bonds to Glu568 and Asp604 of ARD (Fig. 1f). The main-chain O of H4 Arg19 forms a hydrogen bond with Nε1 of Trp563 and its side chain forms contacts with Cys601 and Gly595 of ARD (Fig. 1c). Interactions with the key residue H4 Lys20 are described in the text (Fig. 1g). The intermolecular contacts spanning the Val21-Leu22-Arg23 segment of H4 include contacts between side chains of H4 Val21 with Tyr560 and Cys561 of ARD (b; Fig. 1c). The main-chain N of H4 Arg23 forms a hydrogen bond with the main-chain O of Asp527 of ARD, while the side chain packs against the side chain of Tyr560 of ARD (b), c, Pull-down of recombinant histones H3–H4 with GST–TONSL ARD wild type or indicated mutants. d, Pull-down of pre-purified MCM2 HBD–H3–H4 tetramer complex with GST–TONSL ARD wild type or indicated mutants. e, Circular dichroism analysis of TONSL ARD wild type and the indicated ARD mutants.
Extended Data Figure 4 | Structural comparison of the ARDs of TONSL and GLP. a, b, Representative view of the TONSL ARD with histone H4 tail (a; this work), and crystal structure of the GLP ARD in complex with histone H3 tail dimethylated at Lys9 (b; ref. 10). Both TONSL ARD and GLP ARD use the concave surface to bind their cognate target H4 tail and H3 tail, respectively. TONSL ARD recognizes H4K20me0 mainly through three strong hydrogen bonds with acidic residues Glu530, Asp559 and Glu568, while GLP ARD recognizes H3K9me2 mainly through an aromatic cage forming by residues Trp839, Trp844, Glu847 and Trp877. c, ITC analysis of TONSL ARD binding to H3K9me1 peptide. d, ITC analysis of TONSL acidic stretch and ARD (amino acids 450–692) with H3K9me1 (amino acids 1–21) and H4 (amino acids 9–25) peptides.
Extended Data Figure 5 | Effect of SET8 and MOF depletion on TONSL chromatin binding. a, Immunoprecipitation of GFP–TONSL from solubilized chromatin of GFP–TONSL U-2-OS cells (one representative experiment out of two is shown). Same exposures are shown for input and immunoprecipitation western blots of H3 and H4K16ac. b, TONSL ARD preference for H4K16ac could be mediated by I599 through hydrophobic association with the K16 acetyl group as I599E ARD mutation preferentially reduces binding to H4K16ac peptides as compared to the unmodified H4 tail. Left, pull-down of GFP–TONSL from cell extracts with biotinylated H4 tail peptides. Right, quantification of the western blot, GFP–TONSL binding to the H4K16ac peptide is shown relative to the unmodified peptide. Means with individual data points are shown (n = 2). c, High-content quantitative imaging of TONSL in pre-extracted U-2-OS cells. Plots show total chromatin-bound TONSL and DAPI intensities in cells treated with control or TONSL siRNA, confirming the specificity of TONSL antibody staining. Each dot represents one nucleus. d–f, Analysis of TONSL chromatin-binding in MOF-depleted (d), SET8-depleted (e) and ionizing radiation (IR)-treated cells (f). Chromatin-bound TONSL was quantified by high content imaging of pre-extracted U-2-OS cells stained for endogenous TONSL. Mean TONSL intensity is shown. AU, arbitrary units. d, e, Knockdown efficiency and expected effect on histone modification were confirmed by western blotting (representative of two experiments). e, f, G1 cells were defined by gating on DAPI and EdU intensity. e, TONSL is not recruited to DNA damage in G1 cells, supporting that TONSL accumulation in SET8-depleted cells is due to lack of H4K20me1 and not DNA damage. Cells were irradiated (1.5 Gy) and analysed 1.5 h later (representative of two experiments). d, f, Error bars indicate s.d.; d, from left, n = 4,920, 2,341, 3,608, 2,917; f, n = 382 (–IR), 523 (+IR).
Extended Data Figure 6 | TONSL binding to chromatin during the cell cycle. a, b, H4K20 methylation levels on new and old histones analysed by NCC-pulse-SILAC (data are extracted from ref. 18). Cells grown in light SILAC medium were released into S phase in heavy medium and pulsed with b-dUTP. Chromatin was fixed, sonicated and b-dUTP-labelled fragments isolated on streptavidine beads by NCC. Histones were isolated and analysed by mass spectrometry for modifications on new (heavy) and old (light) histones. For clarity a 24 h (G1/S) chase time point is included. Error bars indicate s.d.; n = 9 (S), 3 (S/G2, M), 5 (G1), 3 (G1/S). Data for M (old histones) is shown as the mean of n = 2, as light peptides were not detected in one of the three biological replicates. c, H4K20 methylation levels measured by mass spectrometry in synchronized TIG3 fibroblasts. d, Plot of mean EdU and total DAPI intensities from TIG3 fibroblasts as in Fig. 3b, with the intensity of chromatin-bound TONSL shown in the third dimension as a colour gradient. AU, arbitrary units. Each dot represents one nucleus. Note that a population of G2 cells (EdU negative) retain TONSL on chromatin. e, High-content quantitative imaging of pre-extracted U-2-OS cells stained for EdU and TONSL analysed as in Fig. 3b. f, Analysis of TONSL chromatin binding by cellular fractionation. U-2-OS cells released from a nocodazole block were followed by fluorescence-activated cell sorting (FACS) analysis of DNA content and analysed by western blotting of soluble (CSK-Triton extracted) and chromatin (pellet) fractions (representative of two experiments).
Extended Data Figure 7 | See next page for caption.
Extended Data Figure 7 | Analysis of GFP–TONSL localization. 

a, Colocalization analysis of chromatin-bound GFP–TONSL with MCM2 analysed by deconvolution microscopy and measurement of Pearson coefficient in single cells. Error bars indicate s.d., $n = 13$ from two independent experiments. Representative image, Fig. 3c. 

b, c, Representative images for the analysis shown in Fig. 3d. Cells were either pulsed with EdU (40 $\mu$M) for 15 min (b) or synchronized in G1/S and released into S phase in the continuous presence of EdU (5 $\mu$M) (c). Images are representative of b: n = 9 (very early), 16 (early/mid), 10 (mid/late); c: 9 (very early), 27 (early/mid), 36 (mid/late). Scale bar, 5 $\mu$m.

b, EdU and MCM2 staining was used to determine the cell cycle state in asynchronous (asyn) cell populations. 

c, Progression through S phase was followed by FACS analysis of DNA content. 

d, Chromatin-binding of GFP–TONSL analysed by cellular fractionation in inducible U-2-OS cells as quantified in Fig. 3e. C, chromatin; S, soluble. 

e, f, Chromatin-binding analysis as in Fig. 3f. U-2-OS cells conditional for GFP–TONSL ARD wild type (WT) and mutant were directly fixed or pre-extracted to remove soluble proteins. Data are representative of three (e) and two (f) experiments, fields of cells in e are representative of (from left) $n = 16$, 18, 17 and 17 images. Scale bar, 20 $\mu$m. 

f, Chromatin-binding analysis as in Fig. 3f. U-2-OS cells conditional for GFP–TONSL were directly fixed or pre-extracted to remove soluble proteins. Data are representative of three (e) and two (f) experiments, fields of cells in e are representative of (from left) $n = 16$, 18, 17 and 17 images. Scale bar, 20 $\mu$m. 

g, Asynchronous U-2-OS cells conditional for GFP–TONSL were pulsed with 40 $\mu$M EdU for 15 min and soluble proteins were extracted. Representative images of EdU-positive cells are shown ($n = 30$ for wild type and N571A), for the specific patterns of TONSL wild type see Fig. 3d. Scale bar, 5 $\mu$m.
Extended Data Figure 8 | See next page for caption.
Extended Data Figure 8 | TONSL–MMS22L recruitment to damaged DNA. a, Left, ChIP-qPCR analysis of GFP–TONSL recruitment to site-specific DSBs induced by AsiSI, as shown in Fig. 4d but with additional controls. Note that the colours have been changed for clarity. Mean of technical duplicates is shown. Each experiment was normalized to GFP–TONSL wild-type enrichment at DSB-I-80bp. Mean is shown with two-sided Mann–Whitney test; ***P < 0.001; not significant, P > 0.05; n = 24. Two-sided Mann–Whitney analysis of individual experiments gave similar results. b, U-2-OS cells conditional for GFP–TONSL were laser microirradiated. 53BP1 and cyclin B staining was used as markers of DNA damage and cells in S/G2 phase, respectively. Representative of three experiments as quantified Fig. 4e. Filled arrowheads indicate GFP–TONSL recruitment; open arrowheads indicate no recruitment. Scale bars, 10 μm. c, U-2-OS cells transiently transfected with GFP–TONSL wild type or the indicated mutants were laser microirradiated and processed for γH2A.X immunofluorescence. Representative cells are shown (n = 200 cells per condition from two independent experiments). d, U-2-OS cells conditional for GFP–TONSL were laser microirradiated. γH2A.X and RPA staining was used as markers of DNA damage and cells undergoing resection in S/G2 phase, respectively. The percentage of GFP–TONSL cells with recruitment to RPA-positive (+) and RPA-negative (−) laser tracks is indicated. Data are representative of two independent experiments, a total of 118 cells were counted. e, Top, U-2-OS cells conditional for GFP–TONSL wild type and N571A were laser microirradiated. γH2A.X and EdU staining was used as markers of DNA damage and S phase cells, respectively. Bottom, quantification of GFP–TONSL cells with recruitment to laser tracks. Mean with individual data points are shown (n = 2, a total of 138 (wild type) and 174 (N571A) cells were counted). f, H4K20 methylation levels measured by mass spectrometry in synchronized TIG3 cells as in Extended Data Fig. 6c. Cell were released into S phase for 3 h and treated with hydroxyurea (HU; 3 mM) or CPT (1 μM) for 3 h or left untreated (6 h). Mean with individual data points are shown (n = 2). g, Colony formation in cells treated with control or TONSL siRNA and induced to express GFP–TONSL. As shown in Fig. 4f, but including additional mutants. Two cell concentrations in technical triplicate from two (E568A, D559A) or four (wild type, N571A) biological replicates are shown. h, Representation of the complementation analysis from Fig. 4f in a single panel including both CPT-treated and untreated cells. This illustrates that the toxicity of the TONSL ARD mutant is comparable to CPT treatment of cells expressing wild-type TONSL. i, Analysis of GFP–TONSL and MMS22L by cellular fractionation in cells inducible for GFP–TONSL ARD wild type and mutant. Representative experiment of the quantification shown in Fig. 4i.
Extended Data Figure 9 | Similarity of the ARDs in TONSL and BARD1, and protein inputs. a, Superposition of the structures of TONSL ARD and BARD1 ARD (Protein Data Bank accession 3C5R)26. The main residues involved in TONSL ARD interactions with the H4 tail are compared to the corresponding residues of BARD1 ARD. The two ARDs show highly similar topology and conservation of the histone-binding surface. b, Input material of the experiment in Fig. 1h. c, Input material of the experiment in Fig. 1j. d, Spot assay with biotinylated H4 tail (amino acids 14–33) peptides confirming equal input into pull-down reactions. e, Input material of the experiment in Fig. 4b. f, Input material of the NCC experiment in Fig. 4c. Note that because ARD mutation disrupts chromatin binding in the presence and absence of CPT (Figs 3e, f and 4a), GFP–TONSL N517 levels are low in the input chromatin. The NCC experiment in Fig. 4c supports our microscopy-based data (Fig. 4a) and further shows that there is no local accumulation of the GFP–TONSL ARD mutant at damaged forks that could have been missed in our microscopy-based quantification of total TONSL on chromatin.
Extended Data Table 1 | Data collection and refinement statistics

|                        | TONSL ARD - MCM2 HBD - H3/H4 Tetramer Complex |
|------------------------|------------------------------------------------|
| **Data collection**    |                                                |
| Space group            | P3 2 1                                         |
| Cell dimensions        |                                                |
| a, b, c (Å)            | 139.5, 139.5, 72.9                             |
| α, β, γ (°)            | 90, 90, 120                                    |
| Resolution (Å)         | 50-2.43 (2.52-2.43)*                          |
| R_pim (%)              | 3.8 (46.8)                                     |
| I/σI                   | 23.1 (1.8)                                     |
| Completeness (%)       | 99.8 (99.7)                                    |
| Redundancy             | 5.5 (5.5)                                      |
| **Refinement**         |                                                |
| No. reflections (total/unique) | 171,308/31,146                               |
| R_work/ R_free (%)     | 20.1/24.6                                      |
| No. atoms              |                                                |
| Protein                | 2,908                                          |
| MES                    | 12                                             |
| GOL                    | 12                                             |
| Water                  | 87                                             |
| B-factors              |                                                |
| Protein                | 81.8                                           |
| MES                    | 108.5                                          |
| GOL                    | 92.6                                           |
| Water                  | 59.8                                           |
| R.m.s deviations       |                                                |
| Bond lengths (Å)       | 0.009                                          |
| Bond angles (°)        | 1.316                                          |

*Highest-resolution shell is shown in parenthesis. One crystal was used for the data.