53BP1 is a reader of the DNA–damage–induced H2A Lys15 ubiquitin mark

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53BP1 (also called TP53BP1) is a chromatin–associated factor that promotes immunoglobulin class switching and DNA double–strand-break (DSB) repair by non–homologous end joining. To accomplish its function in DNA repair, 53BP1 accumulates at DSB sites downstream of the RNF168 ubiquitin ligase. How ubiquitin recruits 53BP1 to break sites remains unknown as its relocalization involves recognition of histone H4 Lys 20 (H4K20) methylation by its Tudor domain. Here we elucidate how vertebrate 53BP1 is recruited to the chromatin that flanks DSB sites. We show that 53BP1 recognizes mononucleosomes containing dimethylated H4K20 (H4K20me2) and H2A ubiquitinated on Lys 15 (H2AK15ub), the latter being a product of RNF168 action on chromatin. 53BP1 binds to nucleosomes minimally as a dimer using its previously characterized methyl–lysine–binding Tudor domain and a carboxy–terminal extension, termed the ubiquitination–dependent recruitment (UDR) motif, which interacts with the epitope formed by H2AK15ub and its surrounding residues on the H2A tail. 53BP1 is therefore a bivalent histone modification reader that recognizes a histone ‘code’ produced by DSB signalling.

DNA double–strand breaks (DSBs) elicit a cascade of protein recruitment on the chromatin surrounding DNA lesions that regulates DNA damage repair and signalling12. 53BP1 is an important effector of this DSB response, as it promotes repair by non–homologous end joining (NHEJ)3 by opposing DNA end resection4, the initiating step in homologous recombination. In mice, 53BP1 is necessary for immunoglobulin class switching5,6 and dysfunctional telomere fusions7, two processes that rely on NHEJ. Furthermore, 53BP1 deficiency in mice leads to a near–complete reversal of the phenotypes associated with loss of BRCA1, including tumorigenesis4,8. 53BP1 must accumulate on the chromatin surrounding DSBs to accomplish its functions9. At the molecular level, 53BP1 acts as a recruitment platform for RIF1, its effector protein during DSB repair by NHEJ10–13. 53BP1 accumulation at DSB sites, as monitored by formation of ionizing radiation (IR)–induced subnuclear foci, requires the recognition of histone methylation, in particular H4K20me214 for which 53BP1 recruits to the chromatin surrounding DSBs to accomplish its functions14. At the molecular level, 53BP1 acts as a recruitment platform for RIF1, its effector protein during DSB repair by NHEJ10–13. 53BP1 accumulation at DSB sites, as monitored by formation of ionizing radiation (IR)–induced subnuclear foci, requires the recognition of histone methylation, in particular H4K20me214 for which 53BP1 recruits to the chromatin surrounding DSBs to accomplish its functions14.

Identification of the 53BP1 UDR

We reasoned that if the above model was strictly correct, the 53BP1 orthologue from fission yeast, Crb2, should also form IR–induced foci in human cells. Indeed, Crb2 contains a tandem Tudor domain that binds to H4K20me2 (Fig. 1a)15. Crb2 accumulates at DSB sites in an H4K20me2–binding–dependent manner16,17, but fission yeast does not have a recognizable RNF168 homologue, as it arose later during evolution. When expressed in human cells as a GFP fusion, Crb2 failed to form IR–induced foci whereas 53BP1 formed foci that co–localized with γ–H2AX (Fig. 1b). As expected, the accumulation of 53BP1 at DSB sites was dependent on H4K20me2 recognition because the 53BP1 D1521R mutation, which disrupts this activity of the Tudor domain, impaired the ability of 53BP1 to form IR–induced foci (Fig. 1b). The inability of Crb2 to accumulate at DSB sites in human cells was not due to a failure of Crb2 to interact with human H4K20me2, as it associated with human chromatin in a Tudor–dependent manner, as determined by fluorescence recovery after photobleaching (FRAP) (Supplementary Fig. 2a–d) and cellular subfractionation (Supplementary Fig. 2e). These experiments suggested that 53BP1 recruitment to break sites might be largely independent of an increased accessibility of H4K20me2 in damaged chromatin.

These observations provided an opportunity to map the region that endows 53BP1 with the ability to accumulate at DSB sites in an RNF168–dependent manner. We refer to this putative region as the ubiquitination–dependent recruitment (UDR) motif. We thus prepared various chimaeras between Crb2 and the minimal focus–forming region (FFR) of 53BP1, which consists of the Tudor domain flanked by an amino–terminal oligomerization region and a C–terminal extension18,19 (that is, 53BP1 residues 1220–1711; Fig. 1a). We separated the 53BP1(FFR) and Crb2 into three regions that were swapped between the two proteins, in various combinations. The chimaeras prepared are illustrated in Fig. 1c and, to facilitate the identification of the chimaeras, segments were labelled ‘S’ if derived from 53BP1 and ‘C’ if derived from Crb2. Because 53BP1 can oligomerize20, all experiments were carried out in cells depleted of endogenous 53BP1.

The domain–swapping experiments first confirmed that the Crb2 Tudor domain can recognize H4K20me2 in human chromatin, as the Crb2 Tudor domain inserted into the 53BP1(FFR) supported localization to break sites (Fig. 1c, d). Second, introduction of the sequence immediately C–terminal of the 53BP1 Tudor domain into Crb2 (CC5

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segment that is highly conserved among 53BP1 orthologues in organisms that have a recognizable RNF8 pathway (Fig. 1f).

The UDR is required for 53BP1 function

RIF1 is the 53BP1 effector during DSB repair10–13. We therefore examined the contribution of the UDR in promoting RIF1 IR-induced focus formation. We tested eight UDR mutations introduced in a short interfering RNA (siRNA)-resistant 53BP1 vector: the five mutations that affect 53BP1 recruitment and three others (K1613A, D1616A and E1624A) that do not. We also included in these assays 53BP1 and 53BP1(D1521R), our positive and negative controls, respectively. We observed that the mutations that impaired 53BP1 accumulation at DSB sites also abrogated RIF1 foci after IR (Supplementary Fig. 5). These results indicated that the UDR is critical for the function of 53BP1 in the DSB response. In further support of this observation, reconstitution of 53BP1<sup>−/−</sup> murine B cells with either the D1521R or L1619A mutants failed to restore class switch recombination (CSR) from IgM to IgG1, whereas reintroduction of wild-type 53BP1 restored CSR (Fig. 1g and Supplementary Fig. 6). Furthermore, the UDR-defective L1619A mutant was unable to restore resistance to IR-induced DSBs in DT40 53BP1<sup>−/−</sup> cells, or to restrict homologous recombination in BRC1 and 53BP1 co-depleted cells (Supplementary Fig. 7). Together, these results indicate that the UDR is necessary for the biological functions of 53BP1.

53BP1 binds to ubiquitinated nucleosomes

Next, we sought to determine the mechanism by which the UDR promotes 53BP1 recruitment to DSB sites. We first considered that the UDR might increase the affinity of 53BP1 for H4K20me2 due to its location; that is, apposed to the Tudor domain. We expressed GST–53BP1 fusion proteins consisting of the tandem Tudor domain with the UDR might increase the affinity of 53BP1 for H4K20me2 due to its location; that is, apposed to the Tudor domain. We expressed GST–53BP1 fusion proteins consisting of the tandem Tudor domain with the UDR. The migration of molecular mass markers (kDa) is indicated on the left. EV, empty vector; IB, immunoblot. These GST–53BP1 fusion proteins, expressed as a T-shaped construct, were pulled down with a biotinylated H4K20me2 peptide. IB, immunoblot.

Chimaera (Fig. 1c, d) produced a protein that accumulated into IR-induced foci that co-localized with γ-H2AX (Fig. 1c, d). Notably, the accumulation of the CC5 chimaera at DSB sites was dependent on RNF168 (Supplementary Fig. 3a, b), strongly suggesting that sequences C-terminal of the 53BP1 Tudor compose the UDR. We further narrowed down the UDR to the region between residues 1604 and 1631 (Supplementary Fig. 3c–e).

Next, we performed alanine-scanning mutagenesis of the UDR, in the context of 53BP1 (1220–1631), to identify residues that participate in the recruitment of 53BP1 to DNA damage sites. These studies identified five residues (I1617, L1619, N1621, L1622 and R1627) that are conserved among 53BP1 orthologues in organisms that have a recognizable RNF8 pathway (Fig. 1f).

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mutation, which abolishes UDR activity, had no effect on H4K20me2 binding (Fig. 2a), indicating that the UDR does not have an impact on recognition of H4K20me2, at least in the context of a peptide.

An alternative function of the UDR might be that it promotes the interaction of 53BP1 with chromatin. To test this possibility, we prepared poly nucleosome-enriched extracts obtained from micrococcal nuclease digestion of human chromatin. Because RNF168 overexpression can trigger 53BP1 accumulation on chromatin, we used RNF168 overexpressed RNF168 (ref. 25), or that were transfected with an empty vector. RNF168 was recently shown to catalyse a new histone mark, RNF8 (ref. 24), we prepared a set of extracts from cells that either overexpressed RNF168 (Supplementary Fig. 8a). We used an N-terminal fragment of RNF168 dimeric 53BP1 fusion protein and the multimeric nucleosomal arrays. Because the above experiments were carried out with polynucleosomes, we conclude that the interaction between 53BP1 and nucleosomes requires the presence of both H4K20me2 and H2AK15ub.

Whereas RNF168 can ubiquitinate H2A K13 or K15 in vivo and in vitro (Fig. 2), we tested whether 53BP1 displayed selectivity towards K13ub or K15ub. To do so, we assembled H4K20me2-containing NCPs with either H2AK13R or H2AK15R substitutions to leave K15 or K13, respectively, as the only residue ubiquitinated by RNF168 (Fig. 3c). To our surprise, when these ubiquitinated NCPs were used in pull-down assays, we found that the 53BP1 Tudor–UDR protein interacted specifically with NCPs containing H2AK15ub (Fig. 3c). This result indicates that 53BP1 has the ability to discriminate between two closely positioned ubiquitinated lysine residues on H2A.

Molecular basis of H2AK15ub selectivity

One possible cause for the 53BP1 selectivity towards H2K15ub could be the presence of sequence elements in the H2A N-terminal tail that are recognized by 53BP1. We noted that three mutations (K9R, A14S and R17S) were sufficient to convert the sequence surrounding K13 to the H2AK15R mutant into the sequence that normally surrounds H2AK15 (Fig. 4a). We found that the resulting mutant (H2AK15Rm3), when ubiquitinated, bound robustly to the 53BP1 Tudor–UDR module (Fig. 4a). We conclude that additional residues in the H2A N terminus contribute to the binding of 53BP1 to 53BP1 containing nucleosomes.

53BP1 recognizes H2AK15ub

Because the above experiments were carried out with poly nucleosomes, the interactions observed could be the product of avidity between the dimeric 53BP1 fusion protein and the multimeric nucleosomal arrays. Therefore, we tested whether we could detect binding between 53BP1 and fully recombinant monomeric nucleosome core particles (Supplementary Fig. 8a). We used an N-terminal fragment of RNF168 ubiquitinated H2A in an interaction between 53BP1 and histones H2A, H3 and H4 (Fig. 2b). However, in the presence of RNF168, we observed a marked increase in the retrieval of ubiquitinated H2A by the Tudor–UDR protein (Fig. 2b). Together, these results indicated that the UDR may stimulate two modes of interaction between 53BP1 and nucleosomes: one mode that is independent of histone ubiquitination, and which may reflect the constitutive interaction of 53BP1 with chromatin; and a second mode of interaction that is dependent on H2A ubiquitination by RNF168, and which may represent the interaction that leads to 53BP1 accumulation at DSB sites.

Figure 3 | 53BP1 is a bivalent reader of the H4K20me2 and H2AK15ub histone marks. a, Pull-down assays of RNF168 ubiquitinated NCPs containing unmethylated histone H4 and H3 (no me), H4K20me2 or H3K9me2 with GST–Tudor–UDR. Ib, immunoblot. b, Pull-down assays of NCPs ubiquitinated with the indicated E3s by GST–Tudor–UDR. A reaction without E1 (No E1) acts as a negative control. c, GST–Tudor–UDR pull-down assays of the indicated NCPs ubiquitinated with RNF168 (+); a reaction lacking E1 (−) was used as negative control. The migration of molecular mass markers (kDa) is indicated on the left.
We next investigated whether ubiquitin recognition contributed to the 53BP1–NCP interaction. Ubiquitin contains a hydrophobic patch centred on its I44 residue that contributes to most ubiquitin-dependent interactions30, and therefore we sought to test whether the ubiquitin I44 residue was important for 53BP1 recognition of H2AK15ub–NCP complexes. We used chemical ubiquitination by disulphide exchange31 to prepare NCPs that contained H2A ubiquitin on K13 (H2AK13ub), K15 (H2AK15ub) and H2AK15 ubiquitinated with Ub(I44A) (H2AK15ub(I44A); Fig. 4b). Those NCPs were then used in pull-down assays with the Tudor–UDR module. As expected, we found that the interaction was selective for H2AK15ub (Fig. 4b). However, H2AK15ub(I44A)-containing NCPs were unable to be retrieved by 53BP1 (Fig. 4b), suggesting that ubiquitin recognition participates in the interaction of 53BP1 with H2AK15ub.

In aggregate, our results support a model where the Tudor–UDR module comprises two histone-modification-binding domains: the Tudor domain that binds H4K20me2, and the UDR, which may interact with ubiquitin-dependent histone binding modes may be central to the function of 53BP1 as an inhibitor of end resection.

Our experiments also identify the first, to our knowledge, site-specific reader of histone ubiquitination. 53BP1 is likely to be one of many readers that interpret the various histone ubiquitination marks identified so far. Proteins such as ASH2L (for H2BK120ub)32, RNF168 and RNF169 (for H2AK13/K15ub)25 are prime candidates for ubiquitin mark readers. RNF169 presents an attractive case because it acts as a competitive inhibitor of 53BP1 (refs 25, 33). These observations and the identification of 53BP1 as an H2AK15ub reader further emphasize the need to decipher the chromatin modification landscape, its regulation and its interpretation, at sites of DNA damage.

METHODS SUMMARY

Human cell lines were maintained at 37 °C and 5% CO₂ atmosphere whereas the avian DT40 cells were grown at 39.5 °C and 5% CO₂ atmosphere. Immunofluorescence microscopy and fluorescent protein imaging were carried out as described previously12,25. Recombinant protein production and pull-down assays were carried out as described previously33,34.

Full Methods and any associated references are available in the online version of the paper.

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**Author Contributions** A.F.-T. initiated the project, carried out most of the cell biological experiments, found the interaction between ubiquitinated H2A and 53BP1, and contributed to the experimental design and data interpretation. M.D. produced the recombinant nucleosomes. A.F.-T. and M.D. and carried out the recombinant nucleosome pull-down studies. C.E.-D. examined Rif1 focus formation, the role of the UDR in homologous recombination and helped with some immunofluorescence experiments. A.O. carried out the class switching experiments and the DT40 work. A.F.-T., C.C.Y.L. and M.-C.L. helped with FRAP data analysis. A.F.-T. and A.O. both receive post-doctoral fellowships from the CIHR; C.E.-D. is an Ontario Post-doctoral Fellow; J.K.-L. receives a post-doctoral fellowship from the Leukemia and Lymphoma Society; and C.C.Y.L. and M.-C.L. are post-doctoral fellows of the Canadian Breast Cancer Foundation (Ontario Division). D.D. is the Thomas Kierans Chair in Mechanisms of Cancer Development and a Canada Research Chair (Tier 1) in the Molecular Mechanisms of Genome Integrity. Work in the D.D. laboratory was supported by CIHR grant MOP84297 and grant GL-01-010 from the Ontario Research Fund.

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METHODS

Cell culture and plasmid transfection. Cell culture human media were supplemented with 10% fetal bovine serum (FBS) and maintained at 37 °C and 5% CO2 atmosphere. U-2-OS (U2OS) cells were cultured in McCoy’s medium (Gibco). HEK293T and HeLa DR-GFP cells were cultured in DMEM (Gibco). HCT116 Flp-In T-REX Flag and Flag–RNF168 stably transfected cells were cultured in DMEM supplemented with 250 μg ml−1 hygromycin B and 5 μg ml−1 blasticidin. CT116 Flp-In T-REX Flag and Flag–RNF168 stably transfected cell lines were described previously22, U2OS and HEK293T cells were purchased from ATCC and HeLa DR-GFP cells were a gift from the laboratory of R. Greenberg. All cell lines were tested negative for mycoplasma contamination. To induce protein expression in these cell lines, 5 μg ml−1 doxycycline was added to the culture medium for 24 h. DT40 cells were obtained from the laboratory of D. Xu and grown at 39.5 °C, 5% CO2 in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum, 1% chicken serum and 0.1 mM β-mercaptoethanol. Plasmid transfections were generally carried out using Lipofectamine 2000 Transfection Reagent (Invitrogen) or Effectene (Qiagen).

Unless stated otherwise, for microscopy experiments, cells were fixed 1 h after irradiation (10 Gy). The DNA was also counter-stained with DAPI (not shown) and used to trace the outline of the nuclei. Retroviral restitution of 53BP1 in B cells. Class switching to IgG1 was assayed in 53BP1−/− mice primary B cells complemented with 53BP1 (1–1711) and mutants thereof by retroviral delivery. Mature B lymphocytes were isolated from the spleens of two males and one female 8–15-week-old 53BP1−/− C57BL/6 strain 129-Trp53bp1tm1C/J mice by depletion of CD43+ cells using CD43 microbeads (Miltenyi Biotech) according to the manufacturer’s instructions. Mice were obtained from Jackson laboratories. Purified B cells were re-suspended at a concentration of 106 cells ml−1 in the presence of 50 μg ml−1 IL-4 (Prepotech) and 25 μg ml−1 LPS (Sigma-Aldrich) or 1 μg ml−1 agonist anti-CD40 (BD) to allow B-cell proliferation/activation. Retroviral particles were collected from the supernatant of Plat-E packaging cells35 transfected with 10 μg of either retroviral vector pMX constructs. Retroviral supernatants were passed through a 0.45 μm filter and ultracentrifuged at 20,000g at 25 °C for 90 min through a 20% sucrose layer to obtain purified virus. B cells were subsequently infected with the retroviral concentrate in the presence of 8 μg ml−1 polybrene (Sigma-Aldrich) and 20 mM HEPES, pH 7.5 by plate centrifugation. The B-cell medium was subsequently changed and replaced with fresh RPMI medium supplemented with 50 μg ml−1 IL-4 and 25 μg ml−1 LPS or 1 μg ml−1 agonist anti-CD40 to induce class switching to IgG1. CSR was analysed 3 days after infection by flow cytometry as described previously11. Experiments with 53BP1−/− mice (Trp53bp1tm1C/J) were carried out according to regulatory standards and were approved by the Mount Sinai Hospital animal care committee (Protocol AUP 0200a).

Chromatin pull down. HEK293 chromatin-enriched extracts were prepared essentially as described25. Chromatin pull downs were performed with 2.5 μg of recombinant GST-tagged proteins immobilized on glutathione sepharose 4B (GE Healthcare) in chromatin pull-down buffer (CPB: 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM diethritol (DTT)) for 1 h at 4 °C. Pull downs were then washed four times with 1 ml of CPB and eluted in 2× Laemmli SDS–PAGE sample buffer for analysis by immunoblotting.

Plasmids. The GFP–53BP1 expression vector (DPD1910) resistant to siRNA 53BP1 no. 1 (ThermoFisher D-003548-01) was described previously25. The GFP was swapped for mCherry using the KpnI-AccI sites to generate pcDNA5-mCherry-FRT/TO-53BP1 (DPD1905). The 53BP1 deletion vectors (consisting of residues 120–1711, 1220–1631, 1484–1603, 1484–1631 or 1604–1631) were created by inserting PCR-amplified fragments (derived from DPD1910) into the NotI and Apal sites of pcDNA5-GFP-FRT/TO; EcoRI and NotI sites of pcDNA5-Flag-FRT/TO-DmrA (DPD1911) and pcDNA5-HA-FRT/TO-DmrC (DPD1912); and in the BamHI and EcoRI sites of modified pETM-30-02 vector in which the ORF of GST was inserted between the hexahistidine tag and the TEV cleavage site or between the BamHI and PstI sites of pMAL-c2X (New England Biolabs). Mammalian expression vectors for the components of the heterodimerization system were generated by PCR amplification of DmRk (FKBP12) and DmRc (FRB) from plVX-Het-2 and plVX-Het-1 (dIDimerize Inducible Heterodimer System, Clontech), respectively, and by ligation into the BamHI and EcoRI sites of Flag-HA-FRT/TO-DmrA (DPD1913) and Flag-HA-FRT/TO-DmrC (DPD1912). BMP-2 was inserted into the BamHI site of the wild-type BMP vector followed by mutation of the HindIII site to Smal. The BMP-2 vectors were then ligated into the BamHI site of pcDNA5-GFP-FRT/TO or pcDNA5-GFP-NLS-FRT/TO (DPD1916). The source of the BMP-2 coding sequence was the pJk148-Bmp2 plasmid (gift from L.-L. Dru29), Chimaeas of 53BP1 and Crb2 were obtained by annealing overlapping PCR fragments (where 555 = amino acids 1220–1483, 1484–1603, 1604–1631; and 556 = amino acids 1358–357, 358–507, 507–708). The annealed fragments were then ligated into the NotI and Apal sites of pcDNA5-GFP-NLS-FRT/TO (DPD1916). The GST–Crb2 Tudor domain (that is, residues 358–507 of Crb2) alone or fused to 53BP1 UDR (residues 1604–1631), 53BP1 UDR (residues 1604–1631) and the Crb2-Tudor(C) and GST–Tudor(C)–UDR(5) vectors, respectively, were, respectively, constructed by inserting PCR-amplified sequences into the BamHI and EcoRI sites of a modified pETM-30-02 described above. Biological vector expression vectors for histones (His5, human H2A in pET15B, His5, human H2B in pET15B, Xenopus laevis H3 in pET3d and X. laevis H4 in pET3a) were obtained from C. Arrowsmith. The RNFL168 expression vector (residues 1–113; DPD1878) was obtained by PCR amplification of the DPD 1109 (ref. 25) and cloned into pPROEX HTa (Invitrogen) using the BamHI and SpeI sites. The BM11-His (residues 1–108) bacterial expression vector (DPD1886) was obtained by PCR amplification of pGEX-4T1-BMI1 (1–108) and cloned into pET24b (+) using Ndel and XhoI sites. The RINGB (residues 1–116) bacterial expression vector (DPD1887) was obtained by PCR amplification of pET28-MIL-RINGB(1–120) and cloned into pGEX 6p-1 using the BamHI and NotI sites, pGEX-4T1-BMI1 (1–120) and pET28-MIL-RINGB(1–120) were gifts of Y. Tong. The retroviral vector pM-K53BP1 (1–1711) and its D1521R derivative were gifts of A. Nussenzweig. All mutations were introduced by site-directed mutagenesis using QuickChange (Stratagene) and all plasmids were sequence-verified.

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Subcellular fractionation. A cytoplasmic fraction (CYTO) was obtained by collecting HEK293 cells in EBC1 buffer (50 mM Tris- HCl pH 7.5, 100 mM NaCl, 0.5% IGEPA L CA-630, 1 mM EDTA, 1 mM DTT, 1× protease inhibitors— Complete, EDTA-free; Roche). After centrifugation at 1,000g for 15 min at 4 °C, the nuclear pellet was re-suspended and periodically vortexed in EBC2 buffer (50 mM Tris- HCl pH 7.5, 300 mM NaCl, 5 mM CaCl2, 1× protease inhibitors—Complete, EDTA-free; Roche) over 30 min. Following centrifugation at 1,000g for 15 min, the supernatant was harvested as the nuclear soluble fraction (NS). The remaining insoluble chromatin fraction was then solubilized by micrococcal nuclease digestion for 30 min at 30 °C and centrifugation at 1,000g for 15 min; the supernatant was collected as the nuclear soluble fraction (CHR).

RNA interference. All siRNAs used in this study were single duplex siRNAs purchased from Thermofisher. RNA interference (RNAi) transfections (40 nmol) were performed using DharmaFECT 1 (ThermoFisher) or RNAiMax (Invitrogen) in a forward transfection mode, following the manufacturer’s protocol. The individual siRNA duplexes used were: 5′-GAGAGCAGAAGUCCUUA-3′, RNF168 (ThermoFisher, D-00152-04, target sequence: 5′-GAGAGCAGAAGUCCUUA-3′), RBCA1 (ThermoFisher D-00346-01, target sequence: 5′-CAGGUACCCUUCCACAUUU-3′) and non-targeting control siRNA (ThermoFisher, D-001210-02, target sequence: 5′-UAAAGCUAUGAAGAAC-3′). Except when stated otherwise, siRNAs were transfected 48 h before cell processing.

Antibodies. We used the following antibodies: mouse anti-53BP1 (clone 19, BD Biosciences), rabbit anti-53BP1 (A300-273A, Bethyl), mouse anti-γ-H2AX (clone JBW301, Millipore), rabbit anti-γ-H2AX (no. 2577, Cell Signaling Technologies), rabbit anti-RBCL (no. 07-434, Millipore), rabbit anti-KAP1 (A300-274A, Bethyl), goat anti-RIF1 (N20) (sc55979, Santa Cruz), mouse anti-Flag (clone M2, Sigma), rabbit anti-cyclin A (gift from M. Pagan), mouse anti-FA (F7-57, Santa Cruz), rabbit anti-H2A (ab18255, Abcam), rabbit anti-H4 (NP-19404, Novus Biologicals), rabbit anti-H3 (ab1791, Abcam), rabbit anti-H2B (ab1790, Abcam), rabbit anti-H4K20me2 (9759, Cell Signaling Technologies), rabbit anti-GST (sc-459, Santa Cruz), mouse anti-MBP (E8032, NEB), rabbit anti-ubiquitin (Z0458, Dako) and mouse anti-actin (CP01, Calbiochem). Peroxidase-affiniPure goat anti-rabbit IgG (111 035 144, Jackson ImmunoResearch) and HRP-linked sheep anti-mouse IgG (NA931, GE Healthcare) were used as secondary antibodies in immunoblotting. Peroxidase-conjugated goat anti-rabbit IgG (111 035 144, Jackson ImmunoResearch) and HRP-linked sheep anti-mouse IgG (NA931, GE Healthcare) were used as secondary antibodies in immunoblotting.

Fluorescence recovery after photobleaching (FRAP). For FRAP experiments, cells were seeded onto 25-mm round coverslips, transferred to a Chamlide Chamber (Molecular Devices) equipped with a Quorum WaveFX Spinning Disc Confocal System (Quorum Technologies) and imaged using a Cytation4 with a 10× Plan Fluor 0.3 NA objective (ThermoFisher). Cells were photobleached in a 100 ms scan using a 405 nm laser (Coherent). Fluorescence recovery after photobleaching (FRAP). Fluorescence microscopy: Alexa Fluor 488 goat anti-mouse, Alexa Fluor 488 donkey anti-mouse, Alexa Fluor 555 goat anti-mouse, Alexa Fluor 555 donkey anti-rabbit, Alexa Fluor 555 donkey anti-goat, Alexa Fluor 647 donkey anti-mouse (Molecular Probes).

Peptides. The H4K20me2 peptide (H4K20me2: biotin- YGGKAGHRKH-K (me2)-VLRD) was purchased from BioBasic and the H2A1K5ub peptide (biotin-spacer-ARAK(Ub)SRSSR; Spacer = 8-aminoo-3,6-dioxaacanonic acid) was purchased from Lifesensors Inc.

Peptride pull downs were performed by incubating 2.5 µM MBP or GST-tagged 53BP1 proteins with 25 µM of the indicated biotinylated histone H4-derived peptide in peptide pull-down buffer (PPB) (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.05% NP-40, 1% BSA). After 2 h at 4 °C, 10 µl of the pull-down reaction mixture was removed as input control and 10 µl of streptavidin-Dynabeads (Dynal) were added to the pull-down mixture and incubated for an additional 30 min at 4 °C. The Dynabeads were then washed twice with 750 µl PBB, twice with 750 µl of 50 mM Tris–HCl pH 8.0, 150 mM NaCl and were then eluted in 25 µl × 2 Laemmli SDS–PAGE sample buffer for analysis by immunoblotting.

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Gel filtration. Estimation of the GST–53BP1 Tudor–UDR and MBP–53BP1 Tudor–UDR molecular masses in solution was done by gel filtration analysis using a 24-ml Superose column (S200 10–300 GL, (GE Healthcare)) in 50 mM HEPES pH 7.5, 150 mM NaCl. Approximately 400 µg of purified GST or MBP-tagged peptide was injected onto the column. The molecular mass of each sample was estimated according to the elution profile of gel filtration standard molecular weight markers (151–1901, Bio-Rad).

NMR spectroscopy. NMR data were acquired at 25 °C on a 600 MHz Bruker AVANCE III spectrometer equipped with a 1.7 mm TCI CryoProbe. Two-dimensional 1H,15N HSQC (heteronuclear single quantum coherence) spectra were collected for 0.2 mM 15N-ubiquitin in the absence or presence of GST, GST–53BP1–UDR (1604–1631) or CDC34. All NMR samples were prepared in 50 mM HEPES pH 7.5, 150 mM NaCl. Approximately 400 µg of purified GST or MBP-tagged protein was injected onto the column. The molecular mass of each sample was estimated according to the elution profile of gel filtration standard molecular weight markers (151–1901, Bio-Rad).

Mass spectrometry. Electrospray ionization mass spectrometry analysis was performed on an Agilent LC/MSD TOF mass spectrometer. Samples were diluted in 0.1% trifluoroacetic acid before analysis. Deconvolution was performed using Agilent MassHunter workstation software for the analysis of modified histones.