MAD2L2 controls DNA repair at telomeres and DNA breaks by inhibiting 5’ end resection

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Appropriate repair of DNA lesions and the inhibition of DNA repair activities at telomeres are crucial to prevent genomic instability. By fuelling the generation of genetic alterations and by compromising cell viability, genomic instability is a driving force in cancer and ageing. Here we identify MAD2L2 (also known as MAD2B or REV7) through functional genetic screening as a novel factor controlling DNA repair activities at mammalian telomeres. We show that MAD2L2 accumulates at uncapped telomeres and promotes non-homologous end-joining (NHEJ)-mediated repair of deprotected chromosome ends and genomic instability. MAD2L2 depletion causes elongated 3’ telomeric overhangs, indicating that MAD2L2 inhibits 5’ end resection. End resection blocks NHEJ while committing to homology-directed repair, and is under the control of 53BP1, RIF1 and PTIP. Consistent with MAD2L2 promoting NHEJ-mediated telomere fusion by inhibiting 5’ end resection, knockdown of the nucleases CTIP or EXO1 partially restores telomere-driven genomic instability in MAD2L2-depleted cells. Control of DNA repair by MAD2L2 is not limited to telomeres. MAD2L2 also accumulates and inhibits end resection at irradiation-induced DNA double-strand breaks and promotes end-joining of DNA double-strand breaks in several settings, including during immunoglobulin class switch recombination. These activities of MAD2L2 depend on ATM kinase activity, RNFL8, RNFL68, 53BP1 and RIF1, but not on PTIP, REV1 and REV3, the latter two acting with MAD2L2 in translesion synthesis. Together, our data establish MAD2L2 as a crucial contributor to the control of DNA repair activity by 53BP1 that promotes NHEJ by inhibiting 5’ end resection downstream of RIF1.

As the processes underlying telomere-driven genomic instability are not completely understood, we performed a functional genetic screen to identify telomere-induced genomic instability regulators (TIGIRs). The TIGIR screen relies on well-controlled reversible inactivation of telomere component TRF2 by expressing temperature-sensitive TRF2(Leu468Ala) (TRF2ts) in Trf2+/− p53+/− (also known as Terf2+/− Trp53+/−) mouse embryo fibroblasts (MEFs). At the permissive temperature (32°C), the TRF2ts MEFs have intact TRF2-mediated telomere protection, but at non-permissive temperatures (37–39°C) TRF2ts is inactive, causing ATM kinase activation, accumulation of DNA damage response (DDR) proteins and NHEJ-dependent ligation at chromosome ends. In the TIGIR screen (Fig. 1a), prolonged TRF2-inactivation causes TRF2ts cells to irreversibly arrest or die owing to severe chromosome end fusion that drives cells into genomic crisis. However, diminished telomere fusion, such as with RNFL8 or DNA ligase IV deficiency, allows survival and proliferation despite telomere uncapping.

In a duplicate TIGIR screen we assayed 1,976 short hairpin RNAs (shRNAs), targeting 391 genes linked to DDR, for shRNAs that allow TRF2ts cells to survive 12 days of telomere uncapping. Among shRNAs targets substantially enriched in at least two out of three screens were several factors previously shown to control telomere fusion (Extended Data Fig. 1a, b), including ATM, NBS1, RAD50, 53BP1 and RNFL8 (refs 11–15). Remarkably, the most prominent screen hit, also independently recovered in a genome-wide TIGIR screen (our unpublished results), was MAD2L2 (Extended Data Fig. 1a, b). MAD2L2 has no known function at telomeres but acts as a non-catalytic interaction partner of REV1 and REV3 in translesion synthesis. Knockdown of Mad2l2 with independent shRNAs markedly increased the survival of TRF2ts MEFs subjected to telomere uncapping, which was abolished by complementation with exogenous shRNA-resistant human MAD2L2 (Fig. 1b, c and Extended Data Fig. 1c–f). Interestingly, we failed to detect a similar activity for REV1 or REV3 (Extended Data Fig. 1g–i).

Indeed, enhanced survival of MAD2L2-depleted cells after long-term TRF2 inactivation was due to diminished telomere fusion. Mad2l2 knockdown significantly reduced telomere fusions, which was prevented by complementation with exogenous MAD2L2 (Fig. 2a and Extended Data Fig. 2a). Telomeres end with a single-stranded 3’ G-overhang that undergoes DNA-ligase-IV/NHEJ-dependent degradation after loss of TRF2-mediated telomere protection. Analysis of telomeric 3’ G-overhangs showed that MAD2L2 depletion prevents overhang loss at 48 h of TRF2 inactivation, further confirming that MAD2L2 is essential for efficient processing of deprotected telomeres by NHEJ (Fig. 2b and Extended Data Fig. 2b). The defect in NHEJ-mediated telomere fusion in Mad2l2-knockdown cells was not explained by changes in cell-cycle progression (Fig. 1b, 32°C panels, and Extended Data Figs 1e and 2c, d). Moreover, MAD2L2 is essential for sister-telomere fusion after activation of DNA repair in mitosis (Extended Data Fig. 2e). In line

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Figure 2 | MAD2L2 facilitates telomeric G-overhang degradation, NHEJ-promoted telomere fusion, repair and CSR. a, Chromosome fusion in shRNA-transduced TRF2ts MEFs (n = 2–4, mean ± s.e.m., ***P < 0.0002, ****P < 0.0001, Kruskal–Wallis analysis of variance (ANOVA)). b, Telomeric single-stranded (ss) G-overhang quantification in TRF2ts MEFs at 32 °C and after 48 h at 37 °C or 39 °C (n = 4, except Mad2l2 sh4 at 37 °C: n = 1, mean ± s.e.m.). c, Tetraploidy (top) and aneuploidy (bottom) after telomere uncapping in TRF2ts MEFs (n = 2, mean ± s.d.). d, NHEJ-mediated repair in U2OS cells analysed by random plasmid integration (left, n = 3) or an EJ5–GFP reporter containing green fluorescent protein (GFP) (right, n = 6, mean ± s.d.). e, Mad2l2 depletion impairs resolution of DDR foci after irradiation (2 Gy) in HDR-deficient (RAD51-depleted) U2OS cells, indicating defective NHEJ (n = 2, mean ± s.e.m.). f, CSR in shRNA-transduced CH12F3-2 cells (n = 3, mean ± s.d.). Ctrl, control.

Figure 3 | MAD2L2 inhibits end resection and promotes telomere-induced genomic instability in a CTIP- and EXO1-dependent manner. a, Flag–MAD2L2 and 53BP1 localization in TRF2ts MEFs at 32 °C and 39 °C, and in p53+/−MEFs after ionizing radiation (IR). Original magnifications, ×63 with zoom factor 3. b, Immunoblotting for DDR factors in TRF2ts MEFs upon telomere uncapping at 39 °C. c, p-ATM, γH2AX, 53BP1, RIF1 and PTIP–GFP foci in TRF2ts MEFs grown for 0, 3 or 12 h at 39 °C (n = 2, mean ± s.d.). d, e, Representative example (d) and quantification (e) of telomeric single-stranded G-overhang analysis in TRF2ts MEFs grown as specified (n = 3, mean ± s.e.m.). f, Quantification of survival assays of TRF2ts MEFs shRNA-transduced as indicated (n = 2, mean ± s.e.m.). g, Immunoblotting for p-ATR(Ser 428), p-CHK1(Ser 345), p-RPA32(Ser 4/Ser 8), RPA34 and CTIP in TRF2ts MEFs upon telomere uncapping at 39 °C.

with MAD2L2 promoting telomere fusion, chromosomal rearrangement and mis-segregation.3–4 MAD2L2 depletion prevented tetraploidy and aneuploidy after telomere uncapping (Fig. 2c and Extended Data Fig. 2f).

To address whether the requirement of MAD2L2 for NHEJ extends beyond telomeres, we investigated whether MAD2L2 depletion affects end-joining in other settings. Knockdown of human MAD2L2 considerably impaired NHEJ-mediated repair of I-Sce1-induced DNA double-strand breaks (DSBs), NHEJ-mediated random plasmid integration, resolution of irradiation-induced DSBs in homology-directed repair (HDR)-deficient cells and clonogenic survival after irradiation, similar to depletion of the NHEJ-promoting factors 53BP1 and RIF1 (Fig. 2d, e and Extended Data Fig. 3a–c). Moreover, class switch recombination (CSR), a physiological process depending on joining of DNA DSBs by NHEJ,5 was substantially impaired in MAD2L2-depleted cells (Fig. 2f and Extended Data Fig. 3f–i). This CSR defect was not due to defects in cell proliferation or expression of Aid (also known as Aicda) messenger RNA, IgM and IgA germ-line transcripts. Thus, MAD2L2 promotes NHEJ-mediated repair in several contexts.

To investigate how MAD2L2 promotes NHEJ, we first addressed its localization. MAD2L2 accumulated both at TRF2-deprotected telomeres and irradiation-induced DSBs (Fig. 3a), implying a direct activity of MAD2L2 at these sites. Next we examined activation of the DDR. At 3, 6 and 12 h of TRF2 inactivation, both control and MAD2L2-depleted cells activated ATM, phosphorylated H2AX, KAP1 and CHK2 (γH2AX, p-KAP1 and p-CHK2, respectively), and formed p-ATM and γH2AX foci to a similar extent (Fig. 3b, c and Extended Data Fig. 4a, b). Moreover, accumulation of 53BP1, RIF1 and PTIP to uncapped telomeres, as well as irradiation-induced DSBs, was unperturbed in MAD2L2-depleted cells (Fig. 3c and Extended Data Fig. 4a–c). Thus, MAD2L2 is not required for recognition of uncapped telomeres or DSBs as damage, nor for initiation of DDR signalling. However, probably reflecting aberrant processing of deprotected telomeres, we noticed increased DDR signalling at 24 and 48 h of TRF2 inactivation in MAD2L2-depleted cells,
visible as increased H2AX and KAP1 phosphorylation, and H2AX ubiquitination (Fig. 3b).

Next we examined the terminal structure of telomeres in more detail. Analysis of 3’ single-stranded G-overhangs at 6 and 12 h of TRF2 inactivation revealed that in the absence of MAD2L2, telomeres gain considerably longer 3’ single-stranded overhangs. At 12 h, Mad2l2-knockdown cells contained 2.5-fold higher, 3’ exonuclease sensitive overhang signals than control cells without uncapping, and nearly sixfold higher overhang signals than control cells at 12 h of telomere uncapping (Fig. 3d, e and Extended Data Fig. 5a). Overhang increase upon MAD2L2 depletion was observed in both wild-type and DNA-ligase-IV-deficient Mad2l2-knockdown cells (Extended Data Fig. 5b–d), indicating that it is not a passive consequence of lack of NHEJ-mediated overhang degradation, but represents an active role of MAD2L2 in inhibiting 5’ end resection. 5’ DNA end resection is a powerful obstruction to NHEJ by creating DNA ends unsuitable for ligation18, explaining why MAD2L2 depletion im-

5’ end resection involves the combined activities of CTP, MRN, EXO1, BLM and DNA2, and is blocked by 53BP1 and its interaction partners RIF1 and PTIP3,13,19–24. In line with MAD2L2 promoting NHEJ by impeding 5’ end resection, co-depletion of CTP or EXO1 with MAD2L2 partially reversed the increased survival of Mad2l2-knockdown cells undergoing prolonged telomere uncapping (Fig. 3f and Extended Data Fig. 5e–h). Moreover, consistent with the generation of longer single-stranded overhangs that block NHEJ and prime for HDR, Mad2l2-knockdown cells showed increased phosphorylation of the ATR target CHK1 and of the single-stranded DNA-binding protein RPA (Fig. 3g).

In addition, MAD2L2-depleted cells showed increased HDR at telomeres by increased telomere sister-chromatid exchanges (TSE; Extended Data Fig. 6a).

Because the effects of MAD2L2 depletion on end resection, telomere fusion and CSR resemble those of 53BP1, RIF1 and, to a certain extent, PTIP inactivation3,13,19–24, we asked whether MAD2L2 is epistatic with 53BP1, RIF1 or PTIP. Similarly, we addressed the requirement of REV1 and REV3 for the ability of MAD2L2 to control end resection and telomere fusion. Unlike in wild-type or PTIP-deficient MEFs, MAD2L2 depletion did not reduce fusion of TRF2-deprotected telomeres in 53BP1- or RIF1-deficient MEFs (Fig. 4a and Extended Data Fig. 6b–e). However, MAD2L2(Leu186Ala) and MAD2L2(Cys70Arg), defective in REV1 or REV3 interaction, respectively25,26, were still capable of promoting telomere fusion and telomere-driven genomic instability (Fig. 4b and Extended Data Fig. 7a, g, i). Moreover, MAD2L2 depletion resulted in increased RPA phosphorylation after irradiation in wild-type, *Pip*/*Pip*, *Rev*/*Rev* and *Rev*/*Rev* MEFs, but not in *53bp1*/*53bp1* (also known as *Trp53bp1*/*Trp53bp1*) or *Rif1*/*Rif1* MEFs (Fig. 4c). This indicates that MAD2L2 controls end resection and fusion of deprotected telomeres in a 53BP1- and RIF1-dependent, but PTIP-, REV1- and REV3-independent manner. Unanticipated, MAD2L2-depleted *Rif1*/*Rif1* MEFs showed an increased background of chromosome end fusion caused by telomere association between sister chromatids (Fig. 4a and Extended Data Fig. 6d, e). The molecular nature of these sister telomere associations remains to be determined, but they occur irrespective of TRF2 inhibition and potentially reflect replication problems27.

To address the dependencies for MAD2L2 function in DNA repair further, we investigated the requirements for MAD2L2 accumulation to DSBs and NHEJ activity at telomeres. Both DSB accumulation and the ability to promote telomere-driven genomic instability by MAD2L2 require an intact HORMA domain and carboxy terminus, while potential S/TQ (Ser or Thr residues that precede Gln) phosphorylation on Thr 103 appears dispensable (Fig. 4b and Extended Data Fig. 7). Interestingly, while MAD2L2 accumulation to irradiation-induced DDR foci (IRIFs) was independent of PTIP or interaction of MAD2L2 with REV1 or REV3, it clearly depended on ATM kinase activity, RNF8, RNF168, 53BP1 and RIF1 (Fig. 4d, e and Extended Data Figs 7c, e and 8). More specifically, MAD2L2 IRIF accumulation required the heat repeats of RIF1 and the same ATM/ATR phosphorylation sites in 53BP1

![Figure 4](https://example.com/figure4.png)

**Figure 4** MAD2L2 localizes to DSBs, inhibits end resection and promotes telomere-NHEJ in a 53BP1- and RIF1-dependent manner. **a,** Chromosome fusion after TRF2 inhibition in MEFs (n = 2, mean ± s.e.m.). WT (wild type) denotes controls to *53bp1*/*53bp1* and *Rif1*/*Rif1* MEFs. See Extended Data Fig. 6c for *Pip*/*Pip* MEFs. **b,** Chromosome fusion in TRF2ts MEFs (mean ± s.e.m.). EV, empty vector. **c,** p-RPA levels in MEFs (n = 2, mean ± s.e.m.), a.u., arbitrary units. **d,** Irradiation-induced MAD2L2, RIF1 and 53BP1 foci in ATM inhibitor (ATMi)-treated U2OS cells (n = 3, mean ± s.e.m.). **e,** MAD2L2 foci in irradiated shRNA-transduced U2OS cells (n = 2, mean ± s.d.). **f,** MAD2L2 and RIF1 foci in irradiated U2OS cells expressing 53BP1-D8 alleles with wild-type or substituted S/TQ sites (schematic in Extended Data Fig. 9a; n = 2, mean ± s.d.). **g,** MAD2L2 foci in irradiated U2OS cells expressing wild-type RIF1 or RIF1 lacking heat repeats (n = 2, mean ± s.d.). **h,** MAD2L2 and RIF1 foci in irradiated asynchronous, G1 or G2 synchronized RPE cells (n = 3, mean ± s.d.).
that promote RIF1-IRIFs, while those that promote PTP1 IRIFs were dispensable21–24 (Fig. 4f, g and Extended Data Fig. 9a–c). Similarly, MAD2L2 localization to uncapped telomeres required 53BP1 (Extended Data Fig. 8e). In line with dependency on RIF1, whose localization to DSBs is strongly reduced in G2, MAD2L2 foci were also diminished in G2, although less than RIF1 foci (Fig. 4h and Extended Data Fig. 9d). The latter could relate to a proposed role for MAD2L2 in the late steps of HDR28.

In addition to inhibiting end resection, 53BP1, but not RIF1, promotes NHEJ by increasing mobility of uncapped telomeres13,20. Consistent with MAD2L2 acting via end resection control downstream of RIF1, MAD2L2 deficiency did not impair mobility of uncapped telomeres (Extended Data Fig. 10a–c).

Altogether, our data identify MAD2L2 as an important regulator of DNA repair pathway choice that promotes NHEJ at telomeres and DSBs in multiple settings, by inhibiting CTIP- and EXO1-dependent 5' end resection downstream of RIF1 (Extended Data Fig. 10d). Whether MAD2L2 affects regulatory acetylation or phosphorylation of CTIP29, or blocks other end resection factors, awaits further investigation.

MAD2L2 and REV3 together compose Polζ and act with REV1 in translesion synthesis to facilitate DNA replication past blocking lesions12. On the basis of reduced gene conversion and impaired RPA foci, RAD51 foci and DSB resolution in REV1- and Polζ-depleted cells, it has been proposed that the ability of REV1/Polζ to alleviate replication stalling might also be important during HDR synthesis29. Although requiring further characterization, together with the data presented here, this suggests differential roles for MAD2L2 in DSB repair. As shown here, MAD2L2 acts downstream of 53BP1/RIF1 in a REV1/REV3-independent manner to promote NHEJ by inhibiting resection, mostly in G1. In addition, MAD2L2, as part of Polζ, might promote HDR by facilitating replication of damaged DNA formed after resection in G2.

The contribution of MAD2L2 to DNA repair at uncapped telomeres and DSBs suggests that aberrant MAD2L2 expression could have pathological consequences by compromising genome integrity. In this respect it is interesting that in particular MAD2L2, more than REV1 or REV3, is reported to be overexpressed in several cancers and has prognostic significance (ref. 29 and the Oncomine database). Furthermore, our finding that MAD2L2 is essential for CSR identifies MAD2L2 as a potential disease-susceptibility gene for human primary immunodeficiencies.

**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 J.J.L.J., V.B., N.M. and S.S.-B. designed the experiments and analyzed the data. M.H.P. and J.J.L.J. performed the TIGR-screw assay, analyzed the screen results and performed initial validation. V.B., N.M., S.S.-B. and B.A.W. performed the experiments, with the exception of assessment of CSR and sister-telomere fusion upon repair activation in mitosis, which were performed and analyzed by A.O. and D.D. S.S.-B., M.H.P. and J.J.L.J. contributed equally. J.J.L.J. wrote the manuscript. Author Information and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to J.J.L.J. (j.jacobs@nikli.nl).
METHODS

TIGR screen. Try2/−/p53−/− TRFs2s MEFs (in short TRFs2s MEFs) were infected in triplicate with PLKO lentiviral shRNA pools consisting of 1,976 shRNAs selected from the Mouse TRC 1.0 Collection. Cells were infected with a multiplicity of infection below 1 and drug selected on 4 µg ml−1 puromycin. After completion of selection, TRFs2s MEFs were plated in duplicate on 15-cm dishes at 150,000 cells per dish and allowed to adhere at 32 °C. One set of plates was kept at 32 °C for 4 additional days, the other set of plates was subjected to 12 days of telomere uncapping at 39 °C, followed by 0 or 2 weeks recovery at 32 °C, corrected for plating efficiency.

Cell culture, growth assays, flow-cytometry. Try2/−/p53−/− TRFs2s MEFs (TRFs2s MEFs) and Try2/−/p53−/−/Lig4−/− TRFs2s MEFs (TRFs2s Lig4−/− MEFS) were generated from Try2/−/p53−/− TRFs2s (TRFs2s) MEFS. Data Fig. 5h), allowed to adhere overnight at 32 °C. After 23,24 the target sequence of the human Ptpn11 gene was amplified using primers P5_Illuseq (5′-GATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-3′, Illuseq_03_PLKO1_f (5′-AAACCCAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA...
Pulsed-field gel electrophoresis and in-gel detection of telomeric DNA. The gel was denatured and re-hybridized with the same probes to obtain a total telomere signal for the purpose of quantification.

### Repair and clonogenic survival assays.

To quantify the repair of DSBs by NHEJ, U2OS cells transduced with shRNAs were transfected with pEG-FPS (NHEJ), as well as an mCherry expression vector and pCAAGS-Ice-f-Tre2 (ref. 30). Transfections were done using Mirus TransIT LT-1 (Mirus Bio LLC). The fraction of mCherry-positive cells that was GFP-positive was determined 72 h after transfection by flow-cytometry on a Bio-LSR Fortessa using FACSdiva software. Quantification was carried out with FlowJo software (TreeStar).

For random plasmid integration assays 500,000 U2OS cells were seeded in 6-cm dishes 24 h before transfection with 3.9 μg of Ndel-linearized pMSCV-blas-GFP and 0.1 μg of mCherry plasmids using ViaFect (Promega). Transfected cells were trypsinized the following day and seeded in 10-cm dishes at different densities for colony formation. Selection was initiated the following day with blastcidin at 5 μg ml^-1. Cells were fixed with 4% paraformaldehyde 10–14 days after plating and stained with 0.1% crystal violet. Colony counting was performed on a ColCount (Oxford Optronix) and values were normalized for plating and transfection efficiencies.

For clonogenic survival assays U2OS cells were seeded in duplicate in 6-well plates at 100, 200 and 400 cells per well for unirradiated cells, and at 400, 800 and 1,600 cells per well for irradiated cells. Cells were grown for 20 h before γ-irradiation and then fixed after 10–12 days of recovery with 4% paraformaldehyde and stained with 0.1% crystal violet. Colonies with >50 cells were counted on a CellC (Oxford Optronix) and the surviving fraction was determined.

### CSR assays.

Research involving animals was performed in accordance with protocols approved by the animal facility at Toronto Centre for Phenogenomics (Toronto). Primary B cell extraction was performed as described before21. In brief, mature B lymphocytes were isolated from spleens of 8–12-week-old C57BL/6 mice by depletion of CD43+ cells using CD43 microbeads (Miltenyi Biotech) according to the manufacturer’s instructions. Purified B cells were resuspended at a concentration of 10^6 cells per ml in the presence of 25 μg ml^-1 LPS (Sigma-Aldrich) to allow B cell proliferation. Both mouse CH12F3-2 (ref. 31) and primary B cells were infected with lentiviral/retroviral particles obtained from the supernatant of HEK293T cell cultures cotransfected with the lentiviral/retroviral shRNA constructs and the vector expressing MLV Gag-Pol (for retrovirus) or the lentiviral psPAX2 packaging vector with VSV-G envelope (2:1:1 ratio). In brief, the viral supernatant was concentrated at 25,000 g for 90 min at 25 °C before resuspension in RPMI culture medium. Infection was subsequently performed as described23 and followed by selection with puromycin.

IgM to IgA switching was assayed in CH12F3-2 cells activated with 1 μg ml^-1 TGF-β1 (Preprotech), 10 ng ml^-1 IL-4 (Preprotech) and 1 μg ml^-1 anti-CD40 (BD). IgA expression was measured by flow-cytometry using biotin-conjugated anti-mouse IgA antibody (1:200; Ebioscience) followed by an anti-biotin-AP-conjugated donkey anti-mouse antibody (1:1,000; Jackson). The signal-to-noise ratio was determined.

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Following digestion with exonuclease III (10 U μl⁻¹, Promega) for 10 min at room temperature, slides were dehydrated through an ethanol series (70%, 95% and 100%) and incubated sequentially with Cy3-TelG 5'-[TTAGGG]₃-3' and FITC-TelC 5'-[CCCTAA]₃-3' probes at room temperature.

**Telomere mobility.** Telomere mobility analysis was done as described with minor adjustments. TRF2ts cells expressing eGFP-TRF1 and mCherry-BP1-2 (53BP1 amino acids 1220–1711) were seeded on Willco wells and left to attach overnight at 32 °C. Before imaging, cells were incubated at 37 °C for 30 min in Leibovitz's L15 medium inside the Microscope climate chamber. Cells were monitored every minute for 20 min (t = 20 frames) at 37 °C. Five-micrometre Z-stacks at 0.5-µm steps in both eGFP and mCherry channels were obtained with a ×63 1.4 oil objective using ZEN software on an Observer Z1 microscope system (Carl Zeiss). Images were obtained at 2 × 2 binning with 512 × 512 pixels. Tracking of telomeres was performed with FIJI software, cells were registered with the Rigid body option from the StackReg plugin. Particles were then tracked with the particle detector and tracker plugin from Mosaic. Telomeres to track were selected on basis of colocalization with mCherry-BP1-2, and tracked for at least 17 out of 20 frames.

**Database.** To address whether changes in MAD2L2 mRNA expression occur in human cancers we consulted the Oncomine database at https://www.oncomine.org/resource/login.html.

**Statistics.** No statistical methods were used to predetermine sample size. P values were determined by Kruskal–Wallis ANOVA test.

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31. Kato, H. et al. Involvement of RBP-J in biological functions of mouse Notch1 and its derivatives. *Development* 124, 4133–4141 (1997).
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Extended Data Figure 1 | Related to Fig. 1. a, A functional genetic screen for TIGIRs identifies independent shRNAs against Mad2l2 and the previously identified regulators of NHEJ-mediated telomere fusion Atm, Nbs1 (also known as Nbn), Rad50, 53bp1 and Rnf8. Listed are the independent shRNAs enriched >1.5-fold in at least two out of three TIGIR screens, with their average ratio of enrichment over all three screens. Ratios reflect shRNA abundance after 12 days of telomere uncapping at 39°C followed by 4 days recovery at 32°C versus shRNA abundance after growth for 4 days at 32°C. b, Additional shRNAs targeting Mad2l2 or known TIGIRs that were enriched >1.5-fold in one out of three screens, with their ratio. Not shown are additional shRNAs against factors not previously implicated in control of telomere fusion, that were considerably enriched in these TIGIR screens but await validation. c, qRT–PCR analysis of Mad2l2 expression levels in TRF2ts MEFs transduced with four independent shRNAs targeting Mad2l2 and used in Fig. 1b (error bars denote s.d.). d, Survival assay of TRF2ts cells infected with control or Mad2l2 sh4 shRNAs, complemented with empty control or RNAi-resistant Flag-MAD2L2RR and grown as indicated. e, Western blot showing expression of endogenous MAD2L2 and exogenous Flag–MAD2L2 in TRF2ts cells used in d and in Fig. 1c. f, Photograph of shRNA-transduced TRF2ts cells grown for 12 days at 39°C. Scale bar, 100 μm. g, None out of ten shRNAs targeting Rev1 or Rev3 were substantially enriched in any of three independent DDR TIGIR screens. h, shRNA-mediated Rev1 knockdown does not increase survival after prolonged telomere uncapping in survival assays of TRF2ts MEFs. Of note, Rev3 knockdown compromised viability and was therefore not informative in these assays. i, qRT–PCR analysis of mouse Rev1 expression levels of cells shown in h (error bars denote s.d.).
Extended Data Figure 2 | Related to Fig. 2.  

a, Representative metaphase spreads of TRF2ts MEFs transduced as indicated, collected after 24 h at 39 °C for telomere FISH. Original magnifications, ×63. b, Representative telomeric single-stranded G-overhang (ss TTAGGG) and total telomere (total TTAGGG) analysis in TRF2ts MEFs at 32 °C and after 48 h at 37 °C or 39 °C. c, d, Mad2L2 knockdown does not affect cell cycle parameters in TRF2ts MEFs. Cell cycle phase analysis was based on propidium iodide staining of asynchronously growing cells (c), as well as on 1 h incubation with BrdU, followed by detection of BrdU incorporation and propidium iodide staining for DNA content (d) (n = 3, mean ± s.d.). e, MAD2L2 is required for sister-telomere fusion upon activation of DNA repair in mitosis. Sister-telomere fusions were quantified in IMR90 cells expressing exogenous wild-type 53BP1 and RNF8, or 53BP1 (Thr1608Ala/Ser1618Ala) (TASA) and RNF8(Thr198Ala) (TA) mutant alleles, and depleted for endogenous RNF8 and 53BP1, as well as depleted for MAD2L2, RIF1 or PTIP (n = 4). f, Examples of DNA content profiles of TRF2ts cells transduced with the indicated shRNAs, grown at 32 °C, or for 48 h at 39 °C and stained with propidium iodide. Analysis of the fraction of cells with 8N (tetraploid) or >4N (aneuploid) DNA content was done on corresponding dotplots of which the results are shown in Fig. 2c.
Extended Data Figure 3 | Related to Fig. 2. 

a, qRT–PCR analysis of MAD2L2 expression levels in U2OS cells infected with control or MAD2L2 shRNAs, and used in the repair assays shown in Fig. 2d (error bars denote s.d.). b, c, qRT–PCR analysis of RAD51 (b) and MAD2L2 (c) expression levels in RAD51-depleted, E6E7-expressing U2OS cells used in the assays shown in Fig. 2e (error bars denote s.d.). d, Clonogenic survival assays of U2OS cells transduced with non-targeting control or 53BP1, RIF1 or MAD2L2 shRNAs and treated with the indicated doses of ionizing radiation (n = 3–4, mean ± s.e.m.).

e, Western blot analysis of 53BP1, MAD2L2 and RIF1 in U2OS cells transduced with the indicated shRNAs. f, CSR in shRNA-transduced primary B cells (n = 2, mean ± s.d.).

CH12F3-2 B cells and mouse primary B cells transduced with the indicated shRNAs. h, MAD2L2 depletion does not affect cellular proliferation in murine B cells. CH12F3-2 cells transduced with control, 53bp1 or Mad2l2 shRNAs were loaded with CFSE and analysed by flow cytometry at 0, 24 and 48 h after stimulation. Profiles from all time points are plotted in the same histogram.

i, MAD2L2 depletion does not affect the transcription of critical genes implicated in CSR. RT–PCR analysis of Aid (Aicda) mRNA, IgM and IgA germline transcript (GLT) levels using twofold serial dilutions of cDNA made from activated CH12F3-2 B cells transduced with the indicated shRNAs. Gapdh was used as a control for transcript expression.
Extended Data Figure 4 | Related to Fig. 3. a, Representative images of immunofluorescence detection of p-ATM(S1981), γH2AX, 53BP1 and RIF1 in TRF2ts MEFs transduced with control or Mad2l2 shRNAs and grown at 32 °C or for 12 h at 39 °C to induce telomere uncapping. DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI). Original magnifications, ×40.

b, Quantification of the number of p-ATM, γH2AX, 53BP1 and RIF1 foci per cell in TRF2ts MEFs transduced with control or Mad2l2 shRNAs and grown at 32 °C or for 3 and 12 h at 39 °C (n = 2, mean ± s.d.).

c, Quantifications of 53BP1, RIF1 and PTIP foci in U2OS cells transduced with control or MAD2L2 shRNAs, 3 h after 5 Gy (n = 2, mean ± s.d.).
Extended Data Figure 5 | Related to Fig. 3. a, Telomeric single-stranded G-overhang assay of TRF2ts MEFs transduced with control or Mad2l2 sh4 shRNAs, showing that the increase in overhang signal upon Mad2l2 knockdown is due to 3’ terminal sequences because the signal is removed by treatment with Escherichia coli 3’ exonuclease Exo1. b, Mad2l2 knockdown causes increased single-stranded telomeric G-overhang signals in TRF2ts Lig4<sup>−/−</sup> MEFs. c, Quantification of relative telomeric G-overhang signals in TRF2ts Lig4<sup>−/−</sup> MEFs transduced with control or Mad2l2 shRNAs and grown at 32 °C or for 12 or 24 h at 39 °C (n = 2, mean ± s.e.m.). d, qRT–PCR analysis of Mad2l2 expression levels in TRF2ts Lig4<sup>−/−</sup> MEFs infected with control or Mad2l2 shRNA lentivirus (error bars denote s.d.). e, Survival assays of TRF2ts MEFs transduced with control or Mad2l2 shRNAs and subsequently with control, Ctip or Exo1 shRNAs. f, Quantification of the survival assays shown in e, g, qRT–PCR analysis of Ctip and Exo1 expression levels of cells shown in e, f and h and in Fig. 3f (error bars denote s.d.). h, Growth curves at 39 °C of TRF2ts MEFs transduced with non-targeting control or Mad2l2 shRNAs and subsequently with control, Ctip or Exo1 shRNAs (error bars denote s.e.m.).
Extended Data Figure 6 | Related to Figs 3 and 4. a, TSCE analysis in shRNA-transduced TRF2ts MEFs grown at 32 °C or for 12 h at 39 °C to uncap telomeres (n = 2, mean ± s.d., counting >1,000 chromosomes per condition, per experiment). TSCE frequency in control cells is set at 1 (corresponding to an average of 6.9% of chromosomes with a TSCE event). Shown on the right are examples of chromosomes without and with TSCE in cells quantified on the left. Original magnifications, ×363. b, qRT–PCR analysis of Mad2l2 expression levels in 53bp12/2, Rif12/2, Ptip2/2 and Ptip1/1 MEFs used in the chromosome fusion analysis shown in Fig. 4a (error bars denote s.d.). c, Percentage of chromosomes fused upon TRF2 inhibition in the Ptip+/+ MEFs matching with the Ptip−/− MEFs shown in Fig. 4a (n = 2, mean ± s.e.m.). d, Analysis of different types of telomere fusions in Rif1−/− MEFs. Depletion of MAD2L2 in Rif1−/− MEFs does not reduce inter-chromosomal telomere fusions induced by TRF2 inhibition, indicating epistasis. However, irrespective of TRF2 inhibition, MAD2L2 depletion in Rif1−/− MEFs induces association between sister telomeres, causing an increase in total fusions scored for MAD2L2-depleted Rif1−/− MEFs, as also visible in Fig. 4a (n = 2, mean ± s.e.m., >1,300–2,000 chromosomes were analysed per condition, per independent experiment). e, Explanation of scoring different types of telomere fusions shown in d.
Extended Data Figure 7 | Related to Fig. 4. a, Schematic overview of C-terminal and N-terminal deletion mutants of MAD2L2. b, c, Expression analysis in U2OS cells of GFP-tagged wild-type MAD2L2 and C- and N-terminal MAD2L2 deletion mutants (b) and of Flag-tagged wild-type MAD2L2 and MAD2L2(Cys70Arg) and MAD2L2(Leu186Ala) (c). d, Analysis of DDR foci formation of GFP-tagged wild-type MAD2L2 and C- and N-terminal MAD2L2 deletion mutants by immunofluorescence detection of GFP and γH2AX in U2OS at 3 h after ionizing radiation (n = 2 for N-terminals, n = 3 for C-terminals, mean ± s.e.m.). e, Analysis of wild-type, Cys70Arg and Leu186Ala MAD2L2 accumulation into DDR foci by immunofluorescence detection of MAD2L2 and 53BP1 in U2OS at 3 h after ionizing radiation (n = 2, ± s.e.m.). f, g, Expression analysis in TRF2ts MEFs of GFP-tagged MAD2L2 and C- and N-terminal MAD2L2 deletion mutants (f) and of Flag-tagged wild-type MAD2L2 and MAD2L2(Cys70Arg), MAD2L2(Leu186Ala), MAD2L2(Thr103Ala) and MAD2L2(Thr103Asp) (g). h, Quantification of chromosome fusions after 24 h of telomere deprotection at 39 °C in TRF2ts MEFs transduced with control or Mad2l2 shRNAs and complemented with empty vector control or RNAi-resistant GFP-tagged wild-type MAD2L2 and C- or N-terminal MAD2L2 deletion mutants (error bars denote s.e.m.). i, Quantification of survival assays of TRF2ts MEFs transduced with control or Mad2l2 shRNAs and subsequently with empty vector control, wild-type MAD2L2, MAD2L2(Cys70Arg), MAD2L2(Leu186Ala), MAD2L2(Thr103Ala) or MAD2L2(Thr103Asp) retroviruses (n = 2, mean ± s.e.m.).
Extended Data Figure 8 | Related to Fig. 4. a, Representative images of immunofluorescence detection of endogenous MAD2L2 and γH2AX or 53BP1 in U2OS cells transduced with control, 53BP1, RNF8, RNF168, RIF1, PTIP or REV3 shRNA lentiviruses, irradiated with 5 Gy and processed for immunofluorescence after 3 h (quantifications are shown in Fig. 4e).

b, Western blot or qRT–PCR analysis of PTIP, RNF8, RNF168 and REV3 levels in shRNA-transduced U2OS cells (error bars denote s.d.).

c, Quantification and representative images of immunofluorescence detection of γH2AX and GFP–MAD2L2 in Ptip+/+ or Ptip−/− MEFS, 3 h after 5 Gy (n = 2, mean ± s.d.).

d, Representative images of immunofluorescence for 53BP1 and exogenous Flag–MAD2L2 wild-type, Cys70Arg or Leu186AAla mutants in U2OS cells depleted for endogenous MAD2L2 with lentiviral shRNA, processed for immunofluorescence 3 h after 5 Gy (quantifications are shown in Extended Data Fig. 7e).

e, Quantification and representative immunofluorescence images of GFP–MAD2L2 localization to uncapped telomeres in TRF2ts MEFS transduced with control or 53bp1 shRNAs (n = 2, mean ± s.d.). Original magnifications, ×63 with zoom factor 5.
Extended Data Figure 9 | Related to Fig. 4. a, Schematic representation of the 53BP1 alleles with wild-type and substituted S/TQ sites used to address MAD2L2 IRIF localization dependence on 53BP1. Representative immunofluorescence images are displayed that show colocalization of endogenous MAD2L2 with 53BP1-DB-WT and -8A, but not with 53BP1-DB-7A or -28A. Quantifications are provided in Fig. 4f. b, Top, representative immunofluorescence images showing colocalization of endogenous RIF1 with 53BP1-DB-WT and -8A, but not 53BP1-DB-7A or -28A. Quantifications are presented in Fig. 4f. Bottom, representative immunofluorescence images showing colocalization of PTIP–GFP with 53BP1-DB-WT and -7A, but impaired colocalization of PTIP–GFP with 53BP1-DB-8A or -28A. c, Schematic representation of GFP-tagged wild-type RIF1 and GFP-tagged RIF1 lacking the N-terminal HEAT repeats (ΔHEAT), used to address MAD2L2 IRIF localization dependence on the HEAT repeats of RIF1. Representative immunofluorescence images are shown. Quantifications are presented in Fig. 4f. d, Cell cycle phase distributions of the RPE cells used in Fig. 4h to address cell cycle dependence of endogenous RIF1 and MAD2L2 localization to IRIFs (n = 3, mean ± s.d.). Original magnifications, ×63 with zoom factor 5.
Extended Data Figure 10 | Related to Fig. 4. a, Frequency distribution (left) and scatter plot (right) of total distances travelled by uncapped telomeres in control or Mad2l2 knockdown cells. b, qRT–PCR analysis of Mad2l2 expression levels and western blot analysis of 53BP1 proteins levels in TRF2ts cells used in the experiments shown in a and c (error bars denote ± s.d.). c, Distance travelled by 10 representative uncapped telomeres for each condition. While multiple uncapped telomeres in 53BP1-depleted cells have reduced mobility, this is not seen for uncapped telomeres in MAD2L2-depleted cells. d, Model of the role of MAD2L2 in promoting NHEJ.