MAD2L2 (REV7) plays an important role in DNA double-strand break repair. As a member of the shieldin complex, consisting of MAD2L2, SHLD1, SHLD2 and SHLD3, it controls DNA repair pathway choice by counteracting DNA end-resection. Here we investigated the requirements for shieldin complex assembly and activity. Besides a dimerization-surface, HORMA-domain protein MAD2L2 has the extraordinary ability to wrap its C-terminus around SHLD3, likely creating a very stable complex. We show that appropriate function of MAD2L2 within shieldin requires its dimerization, mediated by SHLD2 and accelerating MAD2L2-SHLD3 interaction. Dimerization-defective MAD2L2 impairs shieldin assembly and fails to promote NHEJ. Moreover, MAD2L2 dimerization, along with the presence of SHLD3, allows shieldin to interact with the TRIP13 ATPase, known to drive topological switches in HORMA-domain proteins. We find that appropriate levels of TRIP13 are important for proper shieldin (dis)assembly and activity in DNA repair. Together our data provide important insights in the dependencies for shieldin activity.
MAD2L2, for MAD2-Like2, interacts with a variety of proteins to act in multiple important cellular processes. Initially identified as REV7, MAD2L2 directly interacts with REV3 and, together with two accessory proteins POLD2 and POLD3, forms Polymerase ζ (Polζ). Polζ functions in DNA damage tolerance by working with REV1 in translesion synthesis (TLS)-mediated bypass of replication blocking lesions. In addition, MAD2L2 interacts with the anaphase-promoting complex/cyclosome (APC/C) activator CDH1 to prevent premature mitotic exit, and localizes to mitotic spindles and chromosomes through interactions with CHAMP1, RAN GTPase and others. Moreover, MAD2L2 acts at DSBs and uncapped telomeres, where it forms a complex called "shieldin," with the recently characterized SHLD1, SHLD2, and SHLD3 factors. Shieldin functions in DNA repair by localizing to the exposed DNA ends and counteracting 5’ end-resection. By doing so, shieldin complex members, including MAD2L2, promote DNA repair through non-homologous end-joining (NHEJ) while inhibiting DNA repair through homologous recombination (HR). The mode of action that MAD2L2 employs in these different protein complexes is remarkably similar. MAD2L2 is a relatively small protein and consists mainly of a HORMA domain, originally defined by sequence conservation among the yeast proteins Hop1, Rev7, and Mad2. HORMA binding to an interacting motif in its client protein promotes a striking topological remodeling of the HORMA domain from an "open" to a "closed" conformation of its "safety-belt," a structurally mobile element comprising the last ~50 residues of the protein. This process is best understood for spindle assembly checkpoint (SAC) protein MAD2, where the considerable activation energy to be invested in the structural changes results in the creation of the very stable, albeit temporary, signaling complex. The structural conversion of MAD2 is catalyzed, both at the assembly and the disassembly level, by specialized protein machinery, allowing dynamic control of signaling. As part of that machinery, AAA + ATPase TRIP13 has been shown to convert "closed" MAD2 to "open" MAD2. Core properties of MAD2 signaling are conserved in MAD2L2, including the capture of peptides in a closed conformation and dimerization. Indeed, MAD2L2 was found to entrap peptides of different interactors, including REV3, CHAMP1 and RAN GTP, through its safety-belt. These proteins interact with MAD2L2 through a REV7 Binding Motif (RBM), a consensus sequence defined as ϕϕxPxxPxxP. REV3 contains two of these RBMs, that both are important for the function of Polζ in ICL-repair. Interestingly, MAD2L2 is able to bind to both RBMs on REV3 simultaneously and forms a REV3-mediated homodimer, which is important for TLS.

Within the shieldin complex, MAD2L2 directly interacts with SHLD3 and is abolished by mutations in the MAD2L2 safety-belt region. Hence it resembles the interaction between MAD2L2 and REV3. Interestingly, and again in analogy to the REV3-interaction, SHLD3 contains two RBM motifs. However, different from REV3, only one of the SHLD3 RBMs (RBM2) was found important for shieldin function, raising uncertainty on whether MAD2L2 dimerizes in shieldin. We therefore set out to study the importance of MAD2L2 dimerization in the assembly and function of shieldin. Interestingly, we find that abolishing the ability of MAD2L2 to dimerize prevents MAD2L2 from interacting with SHLD2 and impairs its function in NHEJ. Indeed, a recent report showing the crystal structure of a MAD2L2-SHLD3 complex revealed a MAD2L2 dimer within shieldin. Interestingly, analogous to MAD2, the AAA + family ATPase TRIP13 was recently shown to facilitate the opening of the MAD2L2 safety-belt, thereby affecting shieldin complex formation. Indeed, we find that TRIP13 interacts with MAD2L2 in the shieldin complex. We show that this critically depends on SHLD2-driven MAD2L2 dimerization and SHLD3, and that TRIP13 affects shieldin (dis)assembly and DNA repair. Altogether, our work reveals that appropriate activity of shieldin depends on MAD2L2 dimerization to drive appropriate assembly and disassembly of shieldin, facilitated by regulatory activity by TRIP13.

Results
MAD2L2 forms a dimer mediated by SHLD2 and important for shieldin assembly. To understand to what extent MAD2L2’s ability to dimerize affects its function in shieldin we introduced dimer-breaking mutations in a flag-tagged RNAi-resistant (RR) MAD2L2 cDNA expression construct. We introduced an R124A amino acid substitution, originally used to disrupt homodimerization of MAD2L2 in crystallography studies and known to maintain MAD2L2 safety-belt interactions. Interestingly, and again in analogy to the MAD2 dimerization-surface, we thereby obtained expression constructs encoding for MAD2L2 harboring a single amino acid substitution (MAD2L2-R124A, 1xMut) or three (MAD2L2-K44A/R124A/A135D, 3xMut) (Fig. 1a and Supplementary Fig. 1a). We complemented MAD2L2-depleted cells with the different shRNA-resistant wild-type or mutant MAD2L2 expression constructs and confirmed their expression. As expected based on previous report, the mutations did not impair MAD2L2 protein stability (Fig. 1b). To address whether MAD2L2 dimerization affects shieldin complex formation we focused on the two shieldin proteins that directly interact with MAD2L2: SHLD3 and SHLD2. SHLD3 interacts with MAD2L2 through the MAD2L2 safety-belt, and SHLD2 interacts with MAD2L2-SHLD3 through its N-terminal region. SHLD1, on the other hand, participates in shieldin by interacting with the C-terminal domain of SHLD2. Thus, any impact on the interaction between MAD2L2 and SHLD2 would also affect the participation of SHLD1 in shieldin. We co-transfected 293T cells with epitope-tagged SHLD2, SHLD3, and wild-type MAD2L2 or the different MAD2L2-mutants and immunoprecipitated MAD2L2 in order to assess its interaction with SHLD2 and SHLD3. Interestingly, a single R124A amino acid substitution in MAD2L2 (1xMut) strongly reduced its ability to interact with SHLD2, but in line with previous in vitro data on MAD2L2-R124A, SHLD3 interaction, did not disturb the interaction of MAD2L2 with SHLD3 (Fig. 1c). Moreover, three single amino acid changes in the dimerization domain of MAD2L2 completely disrupted its ability to interact with SHLD2, while only slightly affecting its interaction with SHLD3 (Fig. 1c). This indicates that in vivo MAD2L2 dimerization is necessary for efficient interaction of MAD2L2 with SHLD2, but is largely dispensable for MAD2L2-SHLD3 interaction. Indeed, size-exclusion chromatography (SEC) on purified shieldin proteins indicated that SHLD2 induces the dimerization of MAD2L2 in vitro, suggesting that a dimer of MAD2L2 is present in a full-length shieldin complex interacting with single molecules of SHLD3 and SHLD2 (Fig. 1d and Supplementary Fig. 1b, c). Of note, both the wild-type and MAD2L2 (1xMut) appear as monomers in the SEC experiment shown in Fig. 1d. This is expected as the reported dimerization affinity of MAD2L2 is weak, and proteins were applied in low concentrations.

In MAD2, dimerization is known to accelerate the slow, but spontaneous structural conversion from the open to closed topology required for MAD2 incorporation into the Mitotic Checkpoint Complex MCC. In vitro, MAD2 conversion and...
**MCC assembly requires several hours**. Indeed, when mixing MAD2L2 with SHLD3 we observed similar slow complex assembly. The kinetics of complex assembly were further attenuated when using a dimerization deficient MAD2L2 (1xMut, or 3xMut) (Fig. 1, quantified in f). This could subsequently be partially rescued by forcing dimerization of MAD2L2 (1xMut) through a GST fusion (Fig. 1e, f, see Supplementary Fig. 1d for the GST-MAD2L2 dimer). Altogether, these results indicate that within shieldin, MAD2L2 interacts with SHLD3 as a dimer, and that MAD2L2 dimerization is induced by binding of SHLD2. This is in line with recent structural data showing MAD2L2 as a dimer in the shieldin complex.

**Efficient NHEJ at telomeres and in CSR requires MAD2L2 dimerization.** To address the functional importance of MAD2L2 dimerization in DNA repair, we tested the ability of the different MAD2L2 dimerization-mutants to promote NHEJ. For this we made use of Trf2−/−;p53−/− mouse embryonic fibroblasts (MEFs) harboring a temperature-sensitive allele of the telomeric shelterin component TRF2 (TRF2ts) that provide a system for assessing ligase 4-dependent classical NHEJ at natural chromosome ends following TRF2-inactivation mediated telomere deprotection. While these TRF2ts MEFs grow fine at the permissive temperature of 32 °C, culturing them at non-permissive temperatures (37–39 °C) causes TRF2 to dissociate from...
Fig. 2 MAD2L2 dimerization is needed for MAD2L2-mediated control of NHEJ at deprotected telomeres and MAD2L2-mediated class switch recombination. a, Experimental approach to address telomere deprotection induced NHEJ. b, Schematic overview of the experimental setup used in c–e. c, Chromosomal fusions in TRF2ts MEFs transduced with indicated shRNAs and expression constructs (n = 3 independent experiments, mean ± s.e.m., a minimum of 1500 chromosomes were counted per condition, per experiment). One-way ANOVA with Dunnett’s multiple comparisons test (***p < 0.001; ****p = 0.0002). d, Representative images of metaphases counted in c. Scale bar, 10 μm. e, Survival assay of TRF2ts MEFs used in c–d. Cells were cultured at 32 °C to exclude toxicity of the infected cells, or at 39 °C for 12 days to induce telomere deprotection. Cells were returned to 32 °C for the indicated times, and stained to assess survival. Plates are representative of n = 2 independent biological duplicates, the second biological duplicate is shown in Supplementary Fig. 1e, f. In line with our previous work, control cells expressing endogenous MAD2L2 died of crisis upon prolonged telomere uncapping (Fig. 2b, e and Supplementary Fig. 1e, f). In line with the complete loss of MAD2L2-SHLD2 interaction, the triple MAD2L2K44A/R124A/A135D mutant (3xMut) was completely defective in promoting chromosome end fusion in a background of endogenous MAD2L2 depletion (Fig. 2c, d), indicating that this dimerization mutant cannot support NHEJ at telomeres. These findings were recapitulated in an assay for cell survival upon prolonged telomere uncapping, while MAD2L2 depletion allowed these cells to survive. Consistent with their ability to promote telomere fusion, this survival was abolished by re-introducing full-length wild-type MAD2L2, but not by the single and triple MAD2L2-mutants (Fig. 2e and Supplementary Fig. 1e, f).
In addition, we addressed the ability of the different MAD2L2-mutants to promote NHEJ during immunoglobulin class switch recombination (CSR), a physiological process that relies on classical NHEJ-mediated joining of activation-induced cytidine deaminase-mediated DSBs (Supplementary Fig. 2a for experimental setup). As we demonstrated before\(^3\), MAD2L2-deficient cells are strongly impaired in CSR. This CSR defect could be fully restored by complementing MAD2L2-deficient cells with exogenous wild-type MAD2L2 (WT) or with MAD2L2K44A/R124A (1xMut), but not with MAD2L2K44A/R124A/A135D (3xMut) that lost the capability to interact with SHLD2 in the shieldin complex (Fig. 2f and Supplementary Fig. 2b–d). These findings were further recapitulated by assessing the ability of MAD2L2 to prevent IgH breaks and translocations in CH12F3 cells. While IgH breaks and translocations were strongly increased in MAD2L2-deficient CH12F3 cells, these were restored to normal levels by complementation with wild-type MAD2L2 (WT) or MAD2L2K44A/R124A (1xMut), but not with MAD2L2K44A/R124A/A135D (3xMut), indicating that these residues are critical for proper MAD2L2 function at DNA breaks at the IgH locus (Fig. 2g, h).

Interestingly, our finding that NHEJ during CSR, but not telomere-NHEJ, can still be fully supported by the dimerization-defective MAD2L2 (1xMut), indicates that CSR and telomere-NHEJ have different requirements. These might relate to differences in the underlying biology. We hypothesize that for CSR, in which AID induces a DSB at a defined locus, the low level of shieldin assembly that still takes place with the 1xMut is sufficient to maintain functional AID-DSB repair. CSR might therefore be less sensitive to partial defective shieldin complex, such as with the MAD2L2 (1xMut), compared to telomere-NHEJ in which multiple telomeres get deprotected at the same time. Moreover, as 30–50% of CSR can still occur independently of ligase-4/XRCC4-dependent NHEJ, it is possible that in a situation with partially defective shieldin complex some of the breaks are repaired through alternative end-joining.

Altogether this indicates that the interactions mediated by the K44, R124 and A135 residues on MAD2L2, involved in MAD2L2 dimerization, are critical for the ability of MAD2L2 to function in NHEJ.

**The ATP-ase TRIP13 interacts with shieldin in a MAD2L2 dimerization and SHLD3-dependent manner.** To obtain more insight in shieldin complex assembly we set out to identify MAD2L2 interacting proteins with a potential role in MAD2L2 complex formation. For this, we immunoprecipitated MAD2L2 from HeLa cells depleted for endogenous MAD2L2 and stably expressing GFP-tagged MAD2L2, and analyzed co-precipitating peptides by mass spectrometry. Cells were either left untreated or harvested 3 h after inducing DNA damage by irradiation (IR) with 25 Gy. Aside from SHLD2 and SHLD3, we identified multiple additional interacting proteins (Supplementary Fig. 3a). Among these, the AAA + family ATPase TRIP13 in particular caught our attention. One reason for this being its known interaction with MAD2 and other HORMA-domain containing proteins\(^9\). The other reason being that TRIP13 was among positive hits in the same functional genetic screen for endogenous wild-type MAD2L2 and TRIP13, as well as with endogenous MAD2L2 in cells expressing GFP-tagged ATPase-dead TRIP13EQ (Fig. 3a, b). Interestingly, MAD2L2 dimerization appears critical for interaction with TRIP13. In vitro, MAD2L2 directly interacts with TRIP13, but only when dimerization was induced by a GST-tag on MAD2L2 or by the presence of SHLD2\(^1\)–\(^5\) (Fig. 3c, d, f, also see Supplementary Fig. 1d). In addition, interaction of TRIP13 with MAD2L2 requires full-length SHLD3 to be part of the complex. In absence of full-length SHLD3 the interaction of a MAD2L2 dimer with TRIP13 was only very weak or non-detectable (Fig. 3c, compare lanes 7 and 9). Furthermore, SEC revealed that, analogous to MAD2, a hexamer of ATPase activity deficient TRIP13EQ forms a stable complex with MAD2L2-SHLD3 and MAD2L2-SHLD3-SHLD2\(^1\)–\(^5\) in the presence of ATP in vitro (Fig. 3e, f). Interestingly, while TRIP13 association with MAD2 requires p31\(^{comet}\) as a cofactor\(^39\), the formation of a stable TRIP13-shieldin complex apparently does not need a cofactor. However, p31\(^{comet}\) seems important for the activity of TRIP13 towards shieldin\(^40\).

Altogether, our in vivo and in vitro analysis indicate that TRIP13 interacts with shieldin in a manner requiring MAD2L2 dimerization supported by SHLD2, and SHLD3.

**TRIP13 affects DNA repair.** We next addressed the functional relevance of the interaction between TRIP13 and shieldin. We hypothesized that similar to what is known for MAD2\(^17\)–\(^20\), TRIP13 might facilitate a conformational switch in MAD2L2, providing the energy to open up MAD2L2 and release its interacting partner(s). This would allow MAD2L2 to shuttle between different protein complexes and activities, or facilitate release of shieldin activity after DNA repair has advanced or completed. Moreover, by promoting shieldin-disassembly, TRIP13 could promote HR and inhibit NHEJ at the right place and time. Hence, disturbing such a role by TRIP13 might impact DNA repair. To address this we overexpressed exogenous wild-type (WT) or catalytic inactive (ATPase-dead) TRIP13 (TRIP13EQ\(^2\)), or depleted endogenous TRIP13 in different cell lines. We confirmed effective ablation of TRIP13 and functionality of exogenous wild-type or EQ-mutant TRIP13 expression by making use of the notion that a functional SAC requires TRIP13 ATPase-activity\(^41\) (Supplementary Fig. 4a–e). Indeed, unlike TRIP13 proficient cells, cells completely deficient for TRIP13 failed to arrest in mitosis upon mitotic spindle disruption with nocodazole, indicating a dysfunctional SAC. Exogenous expression of wild-type TRIP13, but not TRIP13EQ\(^2\), restored the ability of TRIP13 knockout cells to arrest in mitosis.

We first investigated if TRIP13 affects DNA end-resection, as MAD2L2 and shieldin factors are known to promote NHEJ and inhibit HR by counteracting DNA end-resection. Interestingly, stable overexpression of wild-type TRIP13, in both HeLa and U2OS cells, resulted in increased IR-induced phosphorylation of the single-stranded DNA binding protein RPA on Ser4/8 (p-RPA), indicative of elevated end-resection (Fig. 4a, b). On the other hand, overexpression of ATPase-dead TRIP13EQ did not, or to much lesser extent, increase p-RPA. As TRIP13 functions as a hexamer, we hypothesize that the overexpression of TRIP13EQ\(^2\) in the presence of endogenous TRIP13 can contribute to the formation of TRIP13-hexamers with partial activity. In addition, IR-induced foci formation by RPA appeared slightly elevated (not reaching statistical significance) upon overexpression of wild-type TRIP13, but not TRIP13EQ\(^2\), in U2OS cells (Fig. 4c). As TRIP13 enhanced DNA end-resection in our assays, we hypothesized that overexpression of TRIP13, by facilitating end-resection, would render cells more efficient in HR. To address this, we treated U2OS cells expressing WT or EQ-mutant TRIP13 with the PARP-inhibitor olaparib and addressed survival, as resistance to PARP inhibition is dependent on HR. Indeed, overexpression of WT TRIP13, but not the EQ mutant, caused enhanced resistance to treatment with olaparib (Fig. 4d and Supplementary Fig. 5a). In line with this, cells overexpressing TRIP13 showed enhanced HR...
Representative of two independent experiments. b Immunoblot of GFP-IP in unirradiated or irradiated (25 Gy+3 h) HeLa cells expressing dox-inducible GFP-tagged TRIP13-EQ. Representative of two independent experiments. c GST-tagged MAD2L2 in complex with full-length SHLD3 (lane 7) interacts directly with TRIP13 while the absence of SHLD3 strongly reduces the interaction with TRIP13 (lane 9). Representative of five independent experiments. d Interaction with TRIP13 is dependent on MAD2L2 dimerization (induced here by a GST-tag, which is known to dimerize61). GST-tagged MAD2L2 in complex with full-length SHLD3 (blue), and TRIP13-MAD2L2-SHLD3 (red). When mixed, the elution-profiles of TRIP13 and MAD2L2-SHLD3 shift to an apparent higher molecular weight, indicative of complex formation. MAD2L2-SHLD3 can interact to both the monomer TRIP13 and the hexamer TRIP13. All experiments were performed in presence of 0.1 mM ATP. Asterisk denotes contaminant. Molecular weights of GST-MAD2L2 is 52 kDa, of SHLD3 is 29 kDa and of GFP-TRIP13 is 75.5 kDa (453 kDa for hexamer). Representative of three experiments. f MAD2L2-SHLD3-SHLD21-95 interacts stably with a TRIP13E253Q hexamer. Coomassie-stained SDS-PAGE gels of SEC profiles of TRIP13-MAD2L2-SHLD3-SHLD21-95. Proteins were mixed in equimolar concentrations. All experiments were performed in the presence of 0.1 mM ATP to induce hexamisation of TRIP13E253Q. Molecular weights are: MAD2L2 = 24 kDa, SHLD3 = 29 kDa, SHLD21-95 = 10.3 kDa, and TRIP13E253Q = 48.5 kDa (291 kDa for hexamer). Representative of five experiments. Source data are provided as a Source Data file.

As we reasoned that also absence of TRIP13 might interfere with proper MAD2L2 function we addressed the consequences of CRISPR/Cas9-mediated TRIP13-deficiency for DNA end-resection in stable polyclonal TRIP13 KO U2OS cells, in clonal TRIP13 KO HeLa and U2OS cell lines, and upon short-term polyclonal depletion of TRIP13 with lentiviral sgRNAs (Fig. 4f–j, Supplementary Figs. 4f and 5c, d, f, g). Interestingly, upon different conditions of long-term TRIP13 inactivation in U2OS and HeLa cells, IR-induced p-RPA levels were increased (Fig. 4g and Supplementary Fig. 5c). IR-induced RPA foci were also increased in both MAD2L2 and TRIP13 KO lines (Supplementary Figs. 4f and 5d). Of note, shRNA-mediated depletion of MAD2L2 could still further enhance p-RPA levels in a TRIP13 KO background (Supplementary Fig. 5e). While this could indicate that shieldin-independent roles of TRIP13 contribute to the increased p-RPA in TRIP13-KO lines, it could also reflect more efficient interference with shieldin function that is efficiency in a DR-GFP reporter assay (Fig. 4e and Supplementary Fig. 5b). Finally, we addressed whether the activity of TRIP13 to enhance end-resection depends on MAD2L2. As expected from earlier work5,6, depletion of MAD2L2 resulted in elevated IR-induced p-RPA at 3 h post IR (Fig. 4f). While overexpression of TRIP13 enhanced p-RPA in MAD2L2-proficient cells, it did not further enhance end-resection in a MAD2L2 KO background, indicating that TRIP13 controls end-resection in a MAD2L2-dependent manner. Thus, opposite of the activity of MAD2L2 that counteracts resection and HR, TRIP13 promoted DNA end-resection and HR when overexpressed. This is in line with the interaction we observed between MAD2L2 and TRIP13, with a regulatory activity of TRIP13 on MAD2L2 that is related to the topological switches it induces in HORMA proteins and with a recent report showing that TRIP13 overexpression promotes shieldin-disassembly30.
potentially not complete with either shRNA-mediated depletion of MAD2L2 (despite being very efficient) or TRIP13 KO alone. Conversely, and in line with previous reported findings, efficient but not full disruption of TRIP13 with independent lentiviral sgRNAs was generally associated with signs of reduced end-resection, showing a slight decrease in p-RPA levels and RPA foci in MEFs and U2OS at 3 or 24 hrs post IR (Fig. 4h, i and Supplementary Fig. 5f, g). As RPA responses were mild and for RPA foci not reaching statistical significance over limited number of replicates, we further addressed the effect of TRIP13 depletion on resection by using a PCR-based resection assay. As expected, the amount of ssDNA was enhanced in absence of MAD2L2. TRIP13 depletion had the opposite effect and decreased the amount of resection (Fig. 4j and Supplementary Fig. 5h). In line with this, TRIP13 depletion also decreased HR-efficiency in a DR-GFP reporter assay (Fig. 4e). So while long-term complete TRIP13 disruption appears associated with signs of elevated end-resection (Fig. 4g and Supplementary Figs. 4f and 5c–e), short-term TRIP13 depletion seems associated with decreased resection (Fig. 4h–j and Supplementary Fig. 5f, g).

Fig. 4 TRIP13 affects DNA repair. a Immunoblot of HeLa cells transduced with wild-type TRIP13 (WT), ATPase-dead TRIP13 (EQ) or GFP-control vector (EV), 3 h post IR (10/25 Gy). HSP90 as loading control. Representative of n = 4. b Immunoblot of U2OS cells transduced as in a (25 Gy+3 h), representative of n = 4. c P-RPA-foci in U2OS transduced as indicated, untreated (Und) or 3 h post IR (5 Gy), normalized to control (EV). Dots represent mean ± s.e.m. of replicates, six biological replicates, except sgTRIP13#2 (n = 3) except “Und” (n = 2). d Relative survival of U2OS transduced as b, c treated with olaparib for 19 days (n = 4 biological replicates, showing mean ± s.e.m., one-way ANOVA, Dunnett’s multiple comparisons test for each concentration, significance shown for EV vs. WT, p = 0.1166 (0.5 μM) and p = 0.0846 (1.0 μM); **p = 0.0007 (1.5 μM)). e DR-GFP (HR) assay in U2OS, shRNAs/sgRNA infected or transfected with untagged-TRIP13 (EV, empty vector control). DSBs were induced with I-SceI, except for “Untr”. Dots show independent replicates, mean ± s.e.m., one-way ANOVA (Dunnett’s) (shRNA/sgRNA-treated cells, n = 3 except “Untr” (n = 2), **p = 0.0006; ns p = 0.0720 (sgNT vs. sgMAD2L2) and p = 0.7091 (sgNT vs. sgTRIP13#2); *p = 0.0332) or unpaired two-tailed t-test (TRIP13 overexpression set, n = 4, *p = 0.0173). f Immunoblot of transduced control (NT, non-target) or MAD2L2 KO U2OS cells, untreated (Und) or 9 h post 10 Gy. g Immunoblot of long-term polyclonal TRIP13 KO U2OS cells, a MAD2L2 KO U2OS clone and NT control cells, harvested 3 h post IR. Representative of n = 5. h Immunoblot of transduced U2OS cells, untreated (0 h) or 3 h and 24 h post IR (10 Gy). Representative of n = 3. i P-RPA-foci in U2OS transduced as in h, untreated (Und) or 3 h + 5 Gy, normalized to sgNT. Dots represent n = 4 individual experiments, except sgTRIP13#2/3 (n = 1), 100–600 cells/condition/replicate, mean ± s.e.m., one-way ANOVA (Dunnett’s) for sgNT, sgMAD2L2, sgTRIP13#1 (ns p = 0.2516; *p = 0.0140). j Quantification of single-stranded DNA in RT-PCR-based end-resection assays in sgRNA-transduced U2OS-DivA cells after DSB induction with 4-hydroxytamoxifen (OHT) or untreated (-). Mean ± s.e.m., n = 6, one-way ANOVA (Dunnett’s) (ns p = 0.7939; *p = 0.0332 (sgNT vs. sgTRIP13#1) and p = 0.0445 (sgNT vs. sgMAD2L2)). Significance; ns, not significant (p ≥ 0.05), *p < 0.05, **p < 0.001. Source data are provided as a Source Data file.
We hypothesize that the observed increase in RPA or p-RPA in long-term TRIP13 KO cells reflects disturbed shieldin complex dynamics, or might be a consequence of shieldin-independent roles of TRIP13, as further eluted to in the discussion below.

Overall, the effects of TRIP13 on resection were less prominent than of MAD2L2, which could well be a reflextion of manipulation of a factor that affects the kinetics of shieldin (dis)assembly being less severe than removing a component of shieldin itself. Also, this would be consistent with MAD2L2 spontaneously but slowly disassembling from its interactors over time without the need for TRIP13, in line with the known spontaneous but very slow disassembly of MAD2L2-SHLD3 and other HORMA interactions20-28.

Given the disturbed end-resection control in TRIP13-depleted cells, we assessed sensitivity to olaparib, both in wild-type cells and in BRCA1-deficient cells that are hypersensitive to olaparib due to HR-deficiency. Efficient depletion of TRIP13 in U2OS or RPE-WT cells did not clearly affect the sensitivity to olaparib (Supplementary Fig. 6a, b). However, both shRNA- and sgRNA-mediated depletion of TRIP13 in BRCA1-deficient RPE2 cells rendered these cells less sensitive to olaparib (Supplementary Fig. 6c, d). Although less pronounced, this effect of TRIP13 depletian resembled that of MAD2L2 depletion in BRCA1-deficient cells, observed in the same assays and as reported before6. As PARP inhibitor resistance upon depletion of MAD2L2 in BRCA1-deficient cells is known to be mediated by restoration of end-resection and HR8, this could indicate that HR is also partially restored in TRIP13-depleted BRCA1-deficient cells. This is in contrast to a recent report showing enhanced sensitivity to PARP inhibitors in TRIP13-depleted BRCA1-deficient SUM149PT cells30 and to the decreased HR and resection in BRCA1-proficic U2OS cells we observed upon TRIP13 depletian. These differences might be due to different cell types used, as cells differ in the amount of free MAD2L2 to engage in different protein complexes, further eluted to in the discussion. They may also relate to the signs of elevated resection we observe with long-term depletion of TRIP13 that might come through in these survival assays of longer duration and might result from impaired shieldin complex dynamics or shieldin-independent effects. Furthermore, as MAD2L2 has a prominent role in ICL-repair, we addressed the sensitivity of TRIP13 KO cells to cisplatin induced ICIs. Interestingly, TRIP13 KO U2OS cells displayed modest sensitivity to cisplatin (Supplementary Fig. 7a). Although this did not reach statistical significance over 3 replicates, it may indicate that TRIP13 to some extent also impacts other MAD2L2-functions, such as in ICL-repair.

Finally, we studied the role of TRIP13 at the level of NHEJ. Overexpression of TRIP13 in TRF2ts MEFs did not significantly affect the amount of chromosome end-to-end fusions upon telomere uncapping (Supplementary Fig. 7b, c), suggesting that it did not interfere with the ability of MAD2L2 to promote telomere-NHEJ. However, depletion of TRIP13 using two different shRNAs significantly reduced the amount of chromosomal fusions, albeit to lesser extent than the depletion of MAD2L2 did (Fig. 5a and Supplementary Fig. 7d). This is in line with TRIP13 being among the hits of the same shRNA screen that originally identified MAD2L2 as positive regulator of NHEJ and relies on cell survival upon prolonged telomere uncapping as proxy for impaired NHEJ at telomeres, as extensive fusion of uncapped telomeres causes p53-deficient cells to die of genomic crisis2. (Supplementary Fig. 5b, c). Interestingly, opposite from the effect on telomere-NHEJ, depletion of TRIP13 raised NHEJ during CSR (Fig. 5b and Supplementary Fig. 7e-h). Moreover, the increased CSR-efficiency was dependent on SHLD1 and MAD2L2 (Fig. 5b), indicating that TRIP13 depletion increases CSR-efficiency through the shieldin complex. Thus, while this clearly indicates that in absence of TRIP13, normal control of NHEJ is perturbed, NHEJ at telomeres and at immunoglobulin loci are affected differently. We hypothesize that this might be due to differences in the biology underlying telomere-NHEJ and CSR, or might be related to additional roles for TRIP13 in the context of telomere-NHEJ, as we will further discuss below.

Together our data indicate that aberrant TRIP13 levels, both too much or too little, interfere with normal control of DNA repair, with the outcome for DNA repair being context dependent.

**TRIP13 controls the assembly of SHLD2/3 at DNA breaks.** As DNA repair related processes under control by shieldin were affected by TRIP13, we hypothesized that aberrant expression of TRIP13 impairs shieldin complex dynamics. To address this we studied shieldin complex formation in the absence of TRIP13. We expressed 3xflag-tagged MAD2L2 in 293T cells depleted for TRIP13 or in control cells (sgNT), followed by MAD2L2-immunoprecipitation and mass-spectrometry analysis of immunoprecipitated complexes (Fig. 6a). Interestingly, while the interaction of MAD2L2 with SHLD1 and SHLD2 was not affected, the interaction of MAD2L2 with SHLD3 seemed enhanced in TRIP13 KO cells, despite the difference not being statistically significant over 3 replicate experiments (Fig. 6a). Since SHLD3 is interacting with MAD2L2 through the MAD2L2 safety-belt, the SHLD3-MAD2L2 interaction is the most likely interaction in shieldin to be affected by TRIP13, that would be expected to be stabilized in the absence of TRIP13. To address this we studied the recruitment of the different shieldin complex members to sites of broken DNA in the presence or absence of TRIP13. For this we expressed GFP-tagged SHLD2 or SHLD3 in U2OS-DSB reporter cells, in which DNA breaks are induced with 4-OHT and shield-1 inducible mCherry-LacI-Fok1 nuclease44, and depleted TRIP13 (Fig. 6b–c and Supplementary Fig. 8a–c). As expected, as shieldin localization to DSBs depends on ATM kinase activity5,7, ATM-inhibitor treatment strongly decreased GFP-SHLD2 and GFP-SHLD3 foci intensity at mCherry-marked DSBs. Interestingly, cells depleted for TRIP13 displayed increased GFP-SHLD3 and GFP-SHLD2 accumulation at DSBs. This illustrates that in absence of TRIP13 accumulation of shieldin at DNA DSBs is increased and indicates that TRIP13 affects the recruitment or the stability of the shieldin complex at DNA breaks.

**Discussion**

MAD2L2 engages in multiple complexes to coordinate essential cellular processes, including TLS by participating in Polζ and DSB repair by being a central component of shieldin. By doing so MAD2L2 affects the development and treatment of human disease, such as Fanconi Anemia and cancer, in which it determines the sensitivity of BRCA1-deficient cancers to treatment with Parp inhibitors. Given these critical roles, understanding the requirements for proper MAD2L2 activity is important. Here, we show how dimerization of MAD2L2 is critical for shieldin complex formation and activity in DNA repair and is promoted by SHLD2. Moreover, MAD2L2 dimerization and the presence of SHLD3 allow the shieldin complex to interact with the ATPase TRIP13, which is important for the appropriate dynamics of shieldin complex assembly and appropriate control of DNA repair reactions.

For the function of MAD2L2 in TLS the formation of a REV3-tethered MAD2L2-homodimer, facilitated by two RBM motifs in REV3, is essential24,25. SHLD3 also contains two putative RBM motifs, however only one was found important for the function of shieldin12,28. Whether the formation of a functional shieldin complex would involve and require dimerization of MAD2L2 was
Fig. 5 TRIP13 affects NHEJ-mediated DNA repair. a Chromosome fusions in TRF2ts MEFs infected with indicated shRNAs, after 24 h of telomere uncapping. Values are relative to control infected cells (shCtrl). Dots represent individual experiments (n = 5, except shTrip13#3 (n = 4), mean ± s.e.m., >1500 chromosomes counted per condition per experiment), one-way ANOVA, Dunnett’s multiple comparisons test (**p = 0.0022; ****p < 0.0001). b CSR is increased in absence of TRIP13, using two different shRNAs, but not in a Shld1− and Mad2l2-null background. Mean ± s.e.m. relative to non-infected WT cells, two-sided Wilcoxon–Mann–Whitney test. n = 10 independent experiments except Shld1−−/− (n = 4), and Mad2l2−−/− (n = 3). ****p < 0.0001; ns p = 0.1143 (shCtrl vs. shTrip13#2) and p = 0.3429 (shCtrl vs. shTrip13#3) for Shld1−−/−; ns p = 1 (shCtrl vs. shTrip13#2) and p = 0.4 (shCtrl vs. shTrip13#3) for Mad2l2−−/−. Significance; ns, not significant (p ≥ 0.05), **p < 0.01, ****p < 0.0001. Source data are provided as a Source Data file.

therefore not immediately obvious. Here we show that MAD2L2 indeed forms a dimer within the shieldin complex. This dimer formation is important for the assembly of shieldin and is stabilized by the SHLD2 component. We find that in absence of MAD2L2 dimerization, SHLD2 cannot participate in the shieldin complex in vivo. This finding explains a previous reported result on a K129A-mutation in MAD2L2 that reduced the interaction of SHLD3-MAD2L2 to SHLD1 and SHLD212, as this mutation is located in the MAD2L2 dimerization domain. In line with its necessity for productive shieldin assembly, we show that MAD2L2 dimerization is essential for the function of MAD2L2 in the repair of deprotected telomeres through NHEJ, as well as the appropriate NHEJ-mediated joining of DSBs in immunoglobulin loci during CSR. While completing this manuscript, the dimerization of MAD2L2 was also revealed in the crystal structure of MAD2L2-SHLD31-64_SHLD21-52, showing that a dimer of MAD2L2 molecules is embraced by a peptide of SHLD329. At the C-terminus of the SHLD31-64 peptide, the RBM forms the canonical safety-belt with the MAD2L2 molecule, while the N-terminus of the peptide forms a novel safety-belt interaction with the other MAD2L2 molecule that is mediated by the SHLD2 peptide. This clarifies why previously only one of the SHLD3 RBMs was found important for shieldin function, while we find here that dimerization of MAD2L2 is critical for its in vivo function in shieldin. In addition, our finding that SHLD2 supports the dimerization of MAD2L2 in vitro, is in line with the proposal from the structural data that SHLD2 primes the non-canonical interaction between the SHLD3 N-terminus and MAD2L2.

Importantly, we show that MAD2L2 dimerization not only promotes interactions within shieldin but also brings MAD2L2/shieldin the opportunity to interact with the TRIP13 ATPase. TRIP13 was recently reported to induce the dissociation of MAD2L2-SHLD3 through opening the MAD2L2 safety-belt and subsequent release of SHLD330, indicating that in analogy to MAD2, TRIP13 regulates MAD2L2 complex disassembly in the context of shieldin. Our initial biochemical reconstitution efforts highlighted surprising differences in the molecular mechanism of TRIP13 on shieldin, when compared to the MCC. In the MCC, p31comet, itself in a permanent closed conformation, is an essential cofactor for MCC binding by bridging MAD2 to other HORMA interactions, enhanced TRIP13 activity dissociates shieldin, thereby dismantling its inhibitory activity to end-resection, while lack of TRIP13 activity stabilizes shieldin. However, we also observed seemingly opposite effects of TRIP13 depletion on DNA repair, such as signs of elevated end-resection in long-term TRIP13 KO cells, olaparib resistance in BRCA1-/RPE cells.
TRIP13 slow down disassembly of shieldin and might therefore interfere with the temporal nature of MAD2L2 complex activity promoting disassembly of MAD2L2 from the different complexes it engages in, MAD2L2 would also be locked in its interaction with partner proteins, such as shieldin, Polζ, CHAMP1 and others. We envision that this would strongly impair the ability of MAD2L2 to shuttle between the different complexes it participates in, reduce or ablate the free pool of MAD2L2 available to engage in specific complexes and activities when needed, and interfere with the temporal nature of MAD2L2 complex activity that potentially underlies normal control of the biological processes it operates in. Hence, by interfering with normal release and temporary action of MAD2L2 containing complexes, TRIP13 deficiency could both enhance and impair MAD2L2 controlled processes. The outcome might also be cell type-dependent as cells differ in amounts of free MAD2L2 and the distribution of and, in line with an earlier notion of impaired NHEJ in TRIP13-deficient cells, impaired telomere-NHEJ. These observations indicate that the consequences of TRIP13 depletion are less straightforward and might reflect a more widespread effect of TRIP13 depletion on MAD2L2 interactions that may enhance shieldin function but may also negatively affect shieldin function, depending on context and read-out. We hypothesize that this is a reflection of MAD2L2 being a bi-stable system in which TRIP13 deficiency can yield two results that differentially impact reactions it operates in. Hence, by interfering with normal release and temporary action of MAD2L2 containing complexes, TRIP13 deficiency could both enhance and impair MAD2L2 controlled processes. The outcome might also be cell type-dependent as cells differ in amounts of free MAD2L2 and the distribution of...
MAD2L2 over different complexes. It might also be process dependent as different processes have different requirements due to the underlying biology. For instance, telomere end-joining relies heavily on ligase 4/XRC4 and on the joining of distant deprotected chromosome ends that need to find each other, while end-joining in CSR can still occur for 30-50% in absence of ligase 4/XRC4 and involves cis-joining events of switch-junctions brought together within CSR centers. Affected the MAD2L2-SHLD1-2/3 complex dynamics through modulation of TRIP13 levels might have different outcomes based on these differences. Cell cycle dependencies might also factor in the different outcomes; as NHEJ is preferred in G1, it is possible that the activity of TRIP13 is mostly important in S/G2 to promote efficient HR by inducing shieldin complex disassembly. In addition, it is also possible that some of the effects observed with TRIP13 deficiency are a consequence of other roles of TRIP13, outside of regulatory activity towards MAD2L2.

Overall, our data support a model in which dimerization of MAD2L2 is crucial for shieldin assembly and allows TRIP13 to control appropriate dynamics and activity of the shieldin complex (Fig. 7). The modulation of the reversible topology switch in the MAD2L2 core structure by TRIP13 is expected to regulate its protein-protein interaction potential and kinetics. We therefore speculate that TRIP13 is part of a larger regulating network that will control cell cycle progression and disassembled in the right phase and at the right time. TRIP13, as an enzymatic regulator of shieldin, has been proposed as an interesting candidate to explore as drug target to modulate Parp inhibitor responses. However, the different outcomes we observed with TRIP13-deficiency and the regulation of multiple MAD2L2- or HORMA-complexes by TRIP13, indicate that this might not be straightforward and warrant thorough further exploration of effectiveness and potentially unwanted consequences.

**Methods**

**Cell culture and survival assays.** HeLa, U2OS, 293T, Phoenix (ATCC), DR-GFP U2OS10, AID-DVDA U2OS31, RPE, BRCA1-/-; RPE52 cells and TRF2(-/-); p53(-/-); TRF2(ile648ala) MEFS (TRF2ts MEFS)37,38 were cultured in Dulbecco’s modified Eagle medium with 10% FBS (Sigma), supplemented with 100 U penicillin, 100 μg ml-1 streptomycin and 2 mM l-Glutamine (Gibco, Life Technologies). CH12.1 (murine)31 cell lines were cultured in RPMI 1640 supplemented with 10% FBS, 100 μM l- penicillin, 100 μg ml-1 streptomycin, 50 μM 2-mercaptoethanol, 1x MEM non-essential amino acids, 1 mM pyruvate and 10 mM HEPES. HeLa, U2OS, RPE, 293Ts, Phoenix or CH12 cells were cultured at 37 °C, TRF2ts MEFS were cultured at the permissive temperature of 32 °C, all with 5% CO2. BRCA1 -/- RPE cells were maintained in a low oxygen (3%) incubator. To induce GEP-TRIP13 expression in HeLa cells, cells were treated with 0.5 μg ml-1 doxycycline for 24 h. To evaluate long-term survival upon prolonged telomere deprotection, TRF2ts MEFS were seeded at 40,000 cells/10 cm plate and from the next day cultured at the non-permissive temperature (39 °C) for 12 days. Cells were then returned to 32 °C for the indicated times, fixed with 4% formaldehyde and stained with 0.1% crystal violet. Crystal violet was extracted with 10% acetic acid and absorption at 595 nm was measured in a Tecan microplate reader (Infinite M200pro, TECAN) for quantification.

**Virus production and constructs.** For retrovirus production, phoenix producer cells were transfected with 20 μg plasmid DNA using standard calcium phosphate-DNA precipitation, and refreshed after 16 and 24 h. Supernatant containing viral particles was collected 48 and 62 h post transfection. For Lenti producer cells, transfection was performed with 4 μg ml-1 polybrene and added to the cells. For lentivirus production, 293T cells were transfected with 10 μg plasmid DNA. TRF2ts MEFS were transduced with pLKO-puro shRNA lentiviruses from Mission library clones (Sigma) targeting mouse Mad2l2 (TRCN0000012846: 5’-CATTCTTCAGAAGGCAGAAAG-3’) or a scrambled control hairpin (5’-CAGACAAATGGAGCAACACCA-3’). Pooled, triplicate experiments, pMSCVblass retroviral vectors were used containing a shRNA-resistant wild-type MAD2L2 (MAD2L2(WT)) with a single flag-tag, described before35, as an empty vector control. The Ri24A, K44A and A135D mutations were generated using the QuikChange Site-Directed Mutagenesis Kit (Agilent). Primers are listed in Supplementary Table 1. For TRIP13 overexpression experiments, cells were transduced with lentiviruses containing plX304astoX-5’, Empty, plX304-AST-V5-GFP, plX304-blast-V5-TRIP13, pMSCVblass or pMSCVblass-untagged hTRIP13. For the ATPasenegative TRIP13 mutant (TRIP13(E253Q)), an E253Q mutation was generated into a pX330-puro or pLentiCRISPRv2 plasmid according to standard protocols: sgTRIP13#1: 5’-CGAGTCGCCAACGGTCCACG-3’; sgTRIP13#2: 5’-CACGTGGACCGTTGGCGACT-3’; sgTRIP13#3: 5’-TGAGTAGCTTTCTAACACTC-3’ for pX330-puro. To obtain stable knockout clones, U2OS cells were seeded at 1 x 105 cells/6-well plate and the next day transfected with the pX330-puro CRISPR gRNA constructs containing the
individuals using TransIT-TL1 Transfection reagent (Mirus) and the next day selected with 4 μg/ml 1 μm puromycin for 3–4 days. Next, cells were seeded in 96-well plates and individual clones were picked and grown out. A polyclonal U2OS TRIP13 KO cell line was made by pooling individual TRIP13 KO clones.

For experiments in FoK DS Breporter cells, SHL2D and SHLD3 expression constructs were cloned into a pMSVChl-eGFP retroviral backbone using Gateway technology (Invitrogen).

**Immunoprecipitations.** Immunoprecipitation was done according to standard protocols and as described before. Briefly, cells were washed with PBS and collected by scraping in 2×SDS sample buffer followed by boiling at 95 °C. Alternatively, cell extracts were made using an Urea-SDS buffer (6 M Urea, 1% SDS, 125 mM NaCl, 25 mM Tris pH 8), collected with a cell scraper, sonicated for 15 s at 20% frequency and stored at −20 °C until further use. Protein concentrations were measured using Pierce BCA Protein Assay (Thermo Scientific) and equal amounts of protein were loaded onto precast 4%–12% Bis-Tris gels (Invitrogen). The following antibodies were used: HSP90 (sc-7947, Santa Cruz, 1:500), MADD2L2 (15977, Santa Cruz, 1:500 and 12683-1-AP, ProteinTech, 1:500), pX330 backbone containing puromycin resistance.

For experiments in FoK DS Breporter cells, SHL2D and SHLD3 expression constructs were cloned into a pMSVChl-eGFP retroviral backbone using Gateway technology (Invitrogen).

**Immunoblotting.** Immunoblotting was performed using standard protocols and as described before. Immunoblotting was performed using standard protocols and as described before.

**Mass spectrometry for MAD2L2 interacting proteins.** Mass spectrometry for MAD2L2 interacting proteins was performed using standard protocols and as described before. Immunoblotting was performed using standard protocols and as described before.

**Mass spectrometry for MAD2L2 interacting proteins.** Mass spectrometry for MAD2L2 interacting proteins was performed using standard protocols and as described before. Immunoblotting was performed using standard protocols and as described before.
were refreshed 3 h after transfection and harvested 72 h post transfection by trypsinization, followed by resuspension in PBS and flow cytometry analysis. Cells were gated for single cells and GFP, and the percentage of GFP-positive cells was quantified (Attune TM N&X Acoustic Focusing Cytometer, thermo Fisher Scientific).

**PCR end-resection assay.** The procedure was carried out as described previously. Briefly, 1.5 x 10^6 AID-AsiE-ER-U2OS (AID-DiAVa) cells were seeded in 10 cm plates 24 h before treatment with 0.3 mM 4-hydroxytamoxifen for 4 h. Genomic DNA was isolated using DNeasy Blood & Tissue Kit with RNase A digestion (Qiagen), treated with RNaseH and digested or mock digested with BanI (New England Biolabs) overnight. BanI was then heat inactivated and 12.5 ng of the gDNA was used in a 10 µl quantitative PCR reaction (QuantiStudio 6 flex Real-Time PCR system, Applied Biosystems) in triplicate with SensiFAST SYBR (Bioline) using primers flanking the BanI site 200 bp from the inducible AsiSI cut site (see Supplementary Table 1). The percentage of ssDNA was calculated as previously described.

**Olaparib and cisplatin sensitivity assays.** RPE or U2OS cells transduced with the indicated constructs were seeded at 1000–5000 cells/well (RPE) or 800 cells/well (U2OS) into 6-well plates. For RPE, cells were seeded with either DMSO or the PARP inhibitor olaparib at the indicated concentrations. The medium was refreshed every 5 days, every condition in each experiment was seeded as a technical duplicate. For U2OS cells, olaparib was added at the indicated concentrations the day after seeding. For cisplatin sensitivity assays, 400 cells/well (U2OS) were seeded into 12-well plates as a technical duplicate for each experiment, and cisplatin was added at the indicated concentrations the day after seeding. After 10–12 days, cells were washed with PBS, fixed with 4% formaldehyde and stained using 0.1% crystal violet. The crystal violet was extracted using 10% acetic acid and quantified by measuring absorbance at 595 nm at a plate reader (Infinity M200pro, TECAN). For every condition, crystal violet intensity was normalized to the untreated (DMSO) control.

**CSR assay in CH12 cell lines.** MAD2L2 WT, MAD2L2 R124A, and MAD2L2 3xMut(K44A/R124A/A135D) vectors were introduced by retroviral infection and CH12 cells were selected with blasticidin (10 µg/mL–1). The aforementioned TRIP13-shRNA and control vectors were introduced into CH12 cells which were then transfected with puromycin (3 µg/mL–1) to select for the puromycin-resistant cells. CH12 cells were selected with puromycin (3 µg/mL–1) and the percentage of GFP-positive cells was quantified (Attune TM NXt Acoustic Focusing Cytometer, thermo Fisher Scientific).

**RT-PCR analysis.** IgA germline transcripts (aGLT) and AID mRNA were quantified as previously described. Briefly, total RNA was isolated from activated CH12 cells using the RNAeasy mini kit (Qiagen) and cDNA was synthesized using the high capacity reverse transcription kit (Applied Biosystems). Semi-quantitative RT-PCR using oligonucleotides described in Supplementary Table 1 was performed 1, 3, and 6 days after transfection. The gels were digitized and quantified using imaging software (eBiosciences) and a Fortessa analyser (BD Biosciences). Viable cells were counted using a Casy cell counter (Roche). CSR and proliferation assays were done in at least three independent experiments.

**DNA FISH on metaphase spreads for IgH translocations.** DNA FISH on metaphase spreads was performed as previously described using BAC probes RP24-134G64 (3’ IgH C) and RP24-38617 (3’ IgH V) and Xcloning Mouse Chromosome 12 (orange) paint from MetaSystems. Metaphases were imaged using a Zeiss Axioimager.Z2 microscope and the Metafer automated capture system (MetaSystems), and counted manually.

**Expression of recombinant proteins and purification.** All recombinant proteins used in biochemical reconstitution of complexes in this study were of human origin. MAD2L2 WT, MAD2L2 R124A, MAD2L2 3xMut and SHLD3 WT were expressed with an N-terminal GST/hexahistidine-MBP fusion-tag from pLIB at 1,200–2,400 (Attune TM NXt Acoustic Focusing Cytometer, thermo Fisher Scientific). Bound proteins were eluted with an imidazole gradient. The fusion-tag was cleaved from MAD2L2 WT, MAD2L2 3xMut and MAD2L2 3xMut using PreScission protease. Protein containing fractions were pooled, and concentrated in 10 kDa MWCO merckem (Merck) and loaded onto a Superdex-75 column (GE Healthcare) in tandem with HitTrap Talon Crude 1 ml column (GE Healthcare). Bound proteins were eluted with buffer B (10 mM HEPES pH 7.5, 0.5 M NaCl, 5% glycerol and 0.5 mM TCEP) for size-exclusion chromatography (SEC). Fractions containing purified MAD2L2 and SHLD3 were concentrated, flash-frozen and stored at –80 °C until use. TRIP13 E253Q, MAD2L2 12Mut, SHLD3 and MAD2L2 WT/11Mut. SHLD3/SHLD2 WT complexes were purified as His6-EGFP, His-MBP fusion and GST fusion constructs respectively from insect cell using biGBac Expression system. Bm12 was then carried out from DH10 beta cells and subsequently used to transfect S9 cells and produce baculovirus. Baculovirus was amplified through three rounds of amplification and used to infect Hi5 cells. Cells infected with the viruses were cultured for 72 h before harvesting. Purification of protein complexes was carried out using above protocol while buffer C (25 mM Tris (pH 8), 0.3 M NaCl, 10% glycerol, 0.5 mM TCEP, 10 mM MgCl2, 1 mM PMSF) was used for His6-EGFP-TRIP13 E253Q. Further polishing of MAD2L2 containing complexes prior SEC and after tag cleavage with PreScission protease was carried out by loading onto a cation-exchange (GE) Resource-5 column (GE Healthcare) equilibrated in 10 mM HEPES (pH 7.5), 50 mM NaCl, 5% glycerol and 0.5 mM TCEP. Elution was carried out using NaCl gradient. Fractions containing purified His6-EGFP-TRIP13E253Q, MAD2L2 12Mut, SHLD3 and MAD2L2 WT/11Mut. SHLD3/SHLD2 WT were concentrated, flash-frozen and stored at –80 °C until use.

**In vitro-blowdow assays.** For MBP-pulldown experiments, 0.25 µM MBP-SHLD3 WT pre-adsorbed on Amylose beads was incubated for indicated time points at 4 °C with eightfold molar excess of GST-MAD2L2 WT/MAD2L2 WT/ MAD2L2 11Mut/MAD2L2 23Mut in buffer B. After two washing steps of 0.5 ml each with buffer B, complexes immobilized on beads were analyzed by 12% SDS-PAGE. The gels were digitized and quantified using Fiji. For TRIP13 pulldown experiments, purified proteins were mixed at 6 µM in PD-buffer containing 25 mM Tris (pH 8), 0.3 M NaCl, 10% glycerol, 10 mM MgCl2, 0.5 mM TCEP, 0.01% Triton-X and 3 mM ATP. After 5-min incubation period, glutathione agarose or amylose beads were added. Beads were washed twice with PD-buffer + 0.1 mM ATP and analyzed by 12% SDS-PAGE.

**Size-exclusion chromatography.** SEC runs were performed on Superdex-200 column (GE Healthcare) equilibrated with buffer C + 0.1 mM ATP. Prior run proteins were incubated for 2 h on ice in buffer C + 2 mM ATP. For GST induced Rev7 dimerization profiles, SEC runs were performed on Superdex-200 column equilibrated with buffer B. Fractions collected from SEC runs were analyzed by 12% SDS-PAGE.

**Statistical analysis and reproducibility.** Statistical analyses were performed with GraphPad Prism using the tests as indicated in the respective figure legends. Significance, ns, not significant (p > 0.05), *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Unless indicated otherwise, all immunoblots are representative of at least two independent experiments, with uncropped blots shown in the Source data file.

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

**Data availability.** The data that support this study are available from the corresponding author upon reasonable request. The mass-spectrometry proteomics data generated in this study have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD026912. Source data are provided with this paper.

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Author contributions
L.d.K. performed mutagenesis of MAD2L2 and TRIP13 cDNAs and conducted all cell-based assays, with contributions from J.S., S.H.P., and I.P. and with the exception of CSR-related experiments, DR-GFP and PCR-resection assays. J.S. generated TRIP13 and MAD2L2 KO cell lines, contributed to mutagenesis of MAD2L2 and TRIP13 cDNAs and performed initial characterization of the effect of TRIP13-deficiency on olaparib sensitivity, RPA foci and RPA phosphorylation. S.H.P. contributed to (replicates of) olaparib assays, IP experiments, western blots, Fok1 nuclease DSB reporter and DR-GFP assays. A.M.T. performed PCR-resection assays and contributed to IP experiments. E.V. and C.L. performed experiments and analyzed data for CSR. L.H. performed mass-spectrometry analysis on IP experiments by I.d.K. and L.D. designed experiments and analyzed the data with I.d.K., B.F., and E.V. J.J.L.J. and I.d.K. wrote the manuscript with input from A.C.F. and L.D.

Competing interests
The authors declare no competing interests.

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